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# BIOLOGICAL BULLETIN

## THE EFFECT UPON DEVELOPING EGGS OF EXTRACTS OF EMBRYOS OF THE SAME SPECIES.[1]

MARY GRACE SPRINGER.

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### INTRODUCTION.

The investigations described in this report are an attempt to isolate formative substances from the early embryological stages in the developing egg of *Arbacia punctulata.* Upon the hypothesis of formative stuffs, egg extracts should show the presence of such materials, if under the conditions of the experiment they are isolable. Since development is conceived of as a series of exceedingly complex reactions involving both chemical and physical factors, the formative substances present in the fertilized egg must be the substances which begin this long sequence of reactions. If by their action and interaction, other and possibly more complex substances are synthesized, then at a given stage in development we should expect to find in the extracts of the larvæ the formative substances of that period, provided of course they go into solution in the solvents used. If such is the case, it seems decidedly important to find out whether by treatment of fertilized eggs with extracts of larvæ at a given stage, it is possible to accelerate the development of the eggs when they reach

[1] Contribution from the Zoölogy Department, Oberlin College and the Marine Biological Laboratory.

that specified stage. The cell walls of the developing eggs presumably must be permeable to the formative stuffs present in the solution, if any effect is to be registered.

The whole of the work was done at the Marine Biological Laboratory at Wood's Hole during the summer of 1921. The writer is indebted to Professor C. G. Rogers for suggesting the problem and for his criticism of the plan of work.

## Methods and Materials.

The animals used in these experiments were the starfish, *Asterias forbesii*, and the sea-urchin, *Arbacia punctulata*. The eggs and sperm of both were secured by shaking the ovaries or testes into a dish of sea water. The starfish eggs were filtered through silk bolting cloth to remove portions of the ovarian tissue. The animals were first washed under a stream of fresh water and all precautions were taken to cleanse thoroughly the glassware and instruments used.

By "egg extract" is meant a suspension of crushed eggs in sea water or other specified solvent. The eggs were centrifuged, the fluid decanted, and the eggs ground up with fine sea sand, free from impurities, and thoroughly washed by the solvent used.

In some experiments the eggs were crushed between glass plates, but this method was not satisfactory. No uncrushed eggs were present in the solutions to invalidate the percentages. Extracts of larvæ were made up in the same manner. To maintain as uniform concentration as possible, a definite proportion of eggs to solvent was kept (1 c.c. eggs to 5 c.c. solvent) but the concentration was probably lowered by the retention of some of the protoplasmic fragments in the sand.

The term "blastula water" or "gastrula water," for example, is understood to mean water in which these larvæ have stood up to that stage in their development. Both the eggs and sperm in any one experiment were derived from one individual each whenever possible. The sperm suspension used was concentrated enough to cause 100 per cent. fertilization.

Data of the exact time of the 2-, 4- and 8-cell cleavages chiefly are noted. Although it is difficult to say just when, in terms of

actual minutes after fertilization, the late gastrula becomes a pluteus for instance, by careful comparison of the culture with the control one can observe any differences in their stages of development.

Two methods of comparing rates of cleavage were employed: the time elapsing between insemination and the first 2-, 4- or 8-cell stage, or the period between insemination and the time the culture showed a 50 per cent. development of a given stage. For obvious reasons the former method was used for the most part, only the time required for making a single observation elapsing between the readings, and, in order that there might be no errors introduced merely by the arrangement of the culture dishes, the order of readings was frequently reversed. Equal quantities of eggs from the same suspension were used for each culture to insure an equal opportunity for oxidation and presumably then an equal concentration of $CO_2$ in the water. The cultures were kept in flat-bottomed finger bowls and were stirred frequently. During the season the temperature of the room varied from 21° to 23.5° C.; the variation in the temperature of the sea water in the circulation as recorded each day was found to be from 18.1° C. to 20.5° C. The pH of the sea water was tested each day and was found to be very constant—8.2 or at a few times 8.0 when thymol blue was the indicator used. Whenever the quantity of extract was sufficient to admit of its being tested, the pH was found to be 8.0 or slightly more acid. It did not appear that there was a characteristic difference between the degrees of acidity noted in the extracts made of larvæ in different stages of development.

A new supply of animals was brought into the laboratory each day. These were kept in an aquarium placed on a cement water table with a stream of fresh water running into the aquarium constantly.

The basis of comparison between a normal and a retarded culture in all experiments was (1) rate of development, (2) size, (3) vigor (rate of movement), (4) longevity.

Sample tables are not given for the experiments on the starfish because the rates obtained for both the earlier and late stages were so varied that no definite conclusions can be fairly drawn from them. These variations were doubtless to be explained in part

at least by the fact that toward the end of the breeding season the physiological condition of the eggs was far from uniform. In general it was found that the starfish cultures containing extract showed a slight retardation, a higher degree of cytolysis, and a greater tendency to stop either at the blastula or the gastrula stage.

All the extracts used in these investigations upon *Asterias* eggs were made in a solution of sea water.

## The Effects of Embryological Extracts on the Early Stages of the Sea-Urchin Egg.

The following experiments were all performed on the sea-urchin, *Arbacia punctulata,* to find out if the extracts of various larval stages have any effect on developing eggs of the same species. The eggs of *Arbacia* while more stable, and therefore less desirable than those of *Asterias,* can be obtained in convenient quantities for use in making extracts.

About sixty series of experiments were performed. The method was strictly comparative. It will be noted that the tables show, not only the effect of a given extract, but its effect in varying concentrations as well.

Particular emphasis is laid on the fact that the experiments given in outline in the table are type experiments. Each experiment outlined is one of several very similar experiments unless otherwise stated and is taken as fairly representative of the results obtained in all the experiments of that series, the others of which are omitted to avoid unnecessary and tedious repetition.

*Series 15.*

Table I.

| Culture. | Amount of Sea Water. | Amount of Extract. | Kind of Extract. | Temp. of Sea Water. | pH of Sea Water. | pH of Extract. | Cleavage Rate. | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 2-cell Stage. | 4-cell Stage. | 8-cell Stage. |
| *A*.... | 50 c.c. | 25 c.c. | Gastrula | 18.2° C. | 8.2 | 8.0 | 44 | 92 | 117 |
| *A'*.... | 50 c.c. | 25 c.c. | " | " | " | " | 49 | 97 | 114 |
| *B*..... | 75 c.c. | | | " | " | | 48 | 75 | 98 |
| *B'*.... | 75 c.c. | | | " | " | | 47 | 71 | 96 |

*Note.*—In this particular experiment the figures given under cleavage rate are in terms of minutes which elapsed between insemination and the appearance of the first 2-, 4- or 8-cell stage. It should be noted that the amount of extract used in this series was relatively large. (pH significance discussed later in paper.)

*Later Development and Fate.*—The eggs in cultures *A* and *A'* stopped at the 128–256-cell stage, and a very general cytolysis occurred.

The eggs in *B* and *B'* developed into vigorous normal plutei.

*Conclusion.*—The table shows that there was a definite retardation at the second and third cleavages in the cultures containing extract, and a complete arrest of development at the beginning of the early non-motile blastula stage.

*Series 17.*

TABLE II.

| Culture. | Amount of Sea Water. | Amount of Extract. | Kind of Extract. | Temp. of Sea Water. | pH of Sea Water. | pH of Extract. | Cleavage Rate. | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 2-cell Stage. | 4-cell Stage. | 8-cell Stage. |
| *A*.... | 25 c.c. | 25 c.c. | Blastula Water | 18.4° C. | 8.2 | 8.0 | 50 | 84 | 89 |
| *A'*.... | 50 c.c. | | | " | " | | 50 | 67 | 84 |
| *B*..... | 40 c.c. | 10 c.c. | Motile Blastula | " | " | " | 51 | 68 | 89 |
| *B'*.... | 50 c.c. | | | " | " | | 49 | 66 | 83 |
| *C*..... | 45 c.c. | 5 c.c. | " | " | " | " | 51 | 77 | 81 |
| *C'*.... | 50 c.c. | | | " | " | | 51 | 67 | 83 |
| *D*.... | | 50 c.c. | Blastula Water | " | | | 68 | 106 | 138 |
| *D'*.... | | | | " | " | | 48 | 65 | 84 |

*Note.*—In this experiment the figures given under cleavage rate are in terms of minutes which elapsed between insemination and the presence in the culture of fifty per cent. of the stage indicated.

*Later Development and Fate.*—Development in cultures *B, C,* and to a slight degree in *A,* was definitely retarded, especially between the gastrula and early pluteus stages. In the great majority of cases the gastrulæ did not become plutei at all. The eggs in culture *D* were very much retarded; in fact, few progressed beyond the blastula stage, due probably to lack of oxygen in the water, and possibly to a high concentration of carbon dioxide. Many of the eggs in the controls developed into plutei.

*Conclusion.*—The table shows a very slight retardation in the early cleavage rate of *A*, *B* and *C*, and a greater amount of retardation in the later stages, especially at the early gastrula stage. *D* was very markedly retarded.

A very unusual proportion of exogastrulæ was found in all the cultures. There was even a small proportion in the controls. The reason for this is unknown. It happened that this particular series of experiments was carried on during the hottest days of the summer, but the bowls were kept in the water table, and the temperature of the room was not above 23° C. Later experiments failed to show an equally high percentage of exogastrulæ, although they were carried on under conditions as nearly identical as possible. The only explanation to be offered is that there may have been some variation in the eggs of the particular female used which caused them to evaginate instead of invaginate.

The question of whether these abnormal cultures of exogastrulæ could possibly be influenced to develop normally if extract of larvæ at the same stage was added came up at this point. Of course it is obviously improbable that in making the extract one would at the first trial secure larvæ at the identical point of development of those in the culture. The eggs in the culture were, however, centrifuged, the stale water decanted, and fresh water added. Numerous trials of extracts made up of embryos at different points in the same general developmental stage, and in preceding and succeeding stages were used. In no case did the exogastrulæ proceed with their development.

*Series 32.*

TABLE III.

| Culture. | Amount of Sea Water. | Amount of Extract. | Kind of Extract. | Temp. of Sea Water. | pH of Sea Water. | pH of Extract. | Cleavage Rate. | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 2-cell Stage. | 4-cell Stage. | 8-cell Stage. |
| *A*.... | 50 c.c. | 15 c.c. | Pluteus | 18.6° C. | 8.2 | 8.0 | 62 | 118 | 131 |
| *A'*.... | 65 c.c. | | | " | " | | 55 | 79 | 98 |
| *B*..... | 50 c.c. | 10 c.c. | " | " | " | " | 62 | 96 | 116 |
| *B'*.... | 60 c.c. | | | " | " | | 54 | 77 | 99 |
| *C*..... | 50 c.c. | 5 c.c. | " | " | " | " | 57 | 79 | 120 |
| *C'*.... | 55 c.c. | | | " | " | | 56 | 78 | 96 |
| *D*.... | 30 c.c. | 20 c.c. | Pluteus Water | " | " | | 57 | 84 | 104 |
| *D'*.... | 50 c.c. | | | " | " | | 56 | 80 | 98 |

*Note.*—The cleavage rate is given in terms of minutes between insemination and a fifty per cent. development of the specified stage.

*Later Development and Fate.*—The controls developed faster and more normally than did the other cultures, which were all retarded in the early cleavage stages and at the non-motile blastula stage. All the cultures finally developed into gastrulæ. Some exogastrulæ were found in all the cultures containing extract. No development beyond the late gastrula or very early pluteus stage was observed in the cultures containing extract.

The controls showed normal development.

*Conclusion.*—Definite retardation was noted in the cultures containing extract and total arrest at the late gastrula or early pluteus stages.

*Series 26.*

TABLE IV.

| Culture. | Amount of Sea Water. | Amount of Extract. | Kind of Extract. | Temp. of Sea Water. | pH of Sea Water. | pH of Extract. | Cleavage Rate. | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 2-cell Stage. | 4-cell Stage. | 8-cell Stage. |
| *A*..... | 40 c.c. | 12 c.c. | Water fr. 128–256 cell stage | 18.6° C. | 8.2 | 8.0 | 44 | 77 | 91 |
| *A* .... | 52 c.c. | | " | " | " | | 36 | 70 | 86 |
| *B*..... | 50 c.c. | 5 drops | 128–256 cell stage | " | " | " | 44 | 72 | 85 |
| *B'*.... | 51 c.c. | | " | " | " | | 39 | 70 | 84 |
| *C*..... | 50 c.c. | 10 c.c. | " | " | " | " | 49 | 76 | 93 |
| *C'*.... | 60 c.c. | | " | " | " | | 37 | 68 | 81 |

*Note.*—Under "cleavage rate" is noted the number of minutes which elapsed between insemination and the appearance of the first 2-, 4- or 8-cell stage.

*Later Development and Fate.*—Development to the gastrula stage was nearly parallel in all the cultures. *A* did not develop into very vigorous plutei; *B* showed plutei a trifle smaller than normal, while in *C* were found plutei, but many abnormal types. All the controls had normal plutei.

*Conclusion.*—Slight retardation in cultures containing the extract or the "water."

Several experiments were performed using instead of extracts, water in which eggs had developed. As above specified, "blastula water" is understood to mean water in which eggs have developed to the blastula stage. Details of these experiments are not given because the results do not differ in any way from those obtained by the use of such water as indicated in the above tables. If a relatively large amount of blastula water, *i.e.*, 15–20 c.c. to 50 c.c. of sea water is used, a very slight retardation is noticed when the culture is compared with the control. If only very small amounts of this "water" are employed no perceptible retardation occurs. Since these results seemed to indicate that possibly the retardation in the case of the eggs developing in the blastula water, for example, was due simply to an insufficient supply of O in the water together with increased acidity, boiled sea water was substituted for the blastula water. A very slight retardation was observed—not so evident by any means as that seen in the controls employing blastula water, and quite within the limits of experimental error. This decrease in the degree of retardation, however, may have been due in part, at least, to a decrease in the number of bacteria present in the cultures made up with the boiled sea water.

Since in all the experiments it was quite evident that there was a shorter length of life in the cultures in which extracts were used, an experiment was made using extracts of suspensions which had been boiled previous to being centrifuged, and comparisons drawn

*Series 36.*

TABLE V.

| Culture. | Amount of Sea Water. | Amount of Extract. | Kind of Extract. | Temp. of Sea Water. | pH of Sea Water. | pH of Extract. | Cleavage Rate. | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 2-cell Stage. | 4-cell Stage. | 8-cell Stage. |
| *A*.... | 100 c.c. | 30 c.c. | Blastula (boiled) | 19.7° C. | 8.2 | | 55 | 100 | — |
| *A'*.... | 130 c.c. | | " | " | " | | 45 | 99 | — |
| *B*..... | 100 c.c. | 10 c.c. | " | " | " | | 50 | 95 | — |
| *C*..... | 100 c.c. | 5 c.c. | " | " | " | | 49 | 81 | — |
| *D*..... | 100 c.c. | 30 c.c. | Blastula Water | " | ". | | 52 | 82 | — |
| *E*..... | 100 c.c. | 10 c.c. | " | " | " | | 51 | 80 | — |
| *F*..... | 100 c.c. | 5 c.c. | " | " | " | | 48 | 78 | — |
| *F'*.... | — | 100 c.c. | " | " | " | | — | — | — |

with the results obtained by the use of unboiled extracts. Boiling the suspension to be used for extract was done not only to destroy bacteria, but also to take into account the bare possibility that substances in the larvæ which were evidently not soluble in cold sea water might go into solution in hot water.

*Note.*—Cleavage rate given in terms of minutes which elapsed between insemination and the appearance of the first 2-, or 4-cell stage.

*Later Development and Fate.*—Development in all the cultures was nearly parallel through the blastula stage except in *F'*, in which the eggs failed to segment at all. From the blastula to the pluteus stage the cultures containing extract were very slightly retarded as compared with the control *A'*. There was also a very slight retardation in the cultures containing the specified amounts of blastula water. In all cases, as usual, the retardation resulted in the production of plutei varying slightly from the normal in many respects, but yet producing no one definite type of abnormality.

Boiled extracts of gastrula and early plutei were made also, but the results obtained were entirely similar to those indicated above.

*Conclusion.*—Boiling the extract appears to reduce, if not to eliminate entirely the amount of retardation in the cultures.

Two other methods of making extract in a solution of sea water were tried, since it is obvious that if the extracts were made in a solution of the same osmotic pressure as that in which the eggs developed, that is, sea water, the problem would be much simplified.

The first variation in the method of making the extract was as follows: The eggs were centrifuged, and after the supernatant fluid had been decanted they were placed in a celloidin tube containing distilled water and set in a beaker of sea water. The eggs swelled and burst, presumably setting free substances which, if soluble in water, would dialyze through the celloidin tube into the sea water in the beaker. Extract made in this way was used, but the results obtained were not positive enough to be of value without further experiment. The "double sea water" method was also employed in one series of experiments with equally incon-

clusive results. No results were obtained, however, which were in any way contradictory to those previously noted.

*Series 56.*

An experiment was made, using motile blastulæ for extract, but instead of sea water, distilled water was employed, and the crushed eggs left over night (12 hours) in the distilled water before the extract was added to the cultures.

TABLE VI.

| Culture. | Amount of Sea Water. | Amount of Extract. | Kind of Extract. | Temp. of Sea Water. | pH of Sea Water. |
|---|---|---|---|---|---|
| *A*..... | 50 c.c. | 5 c.c. | Motile blastula | 20.3° C. | 8.2 |
| *A'*..... | 50 c.c. sea water<br>5 c.c. distilled water | — | | " | " |
| *B*..... | 50 c.c. | 3 c.c. | " | " | " |
| *B'*..... | 50 c.c. sea water<br>3 c.c. distilled water | — | | | |
| *C*..... | 50 c.c. | 1 c.c. | " | " | " |
| *C'*..... | 50 c.c. sea water<br>1 c.c. distilled water | — | | " | " |

*Later Development and Fate.*—All the cultures and the controls appeared to reach the 128–256-cell stage at approximately the same time. A reading made four hours and fifteen minutes later showed *A* retarded, non-motile blastulæ; *A'*—blastulæ—a few motile. In *B* and *B'*, there were blastulæ, motile in both, but more vigorous in *B'*. No difference appeared at this stage in *C* and *C'*, in both of which the blastulæ were motile.

In the readings made on the following day, it appeared that *A* had stopped at the non-motile blastula stage. In *A'* were found vigorous gastrulæ. In *B* there were only a very few blastulæ which were moving while *B'* showed very vigorous late gastrulæ. No difference was noted between cultures *C* and *C'*. Both of these showed vigorous late gastrulæ.

*Conclusion.*—The cultures containing an appreciable amount of extract exhibited a retardation at the early non-motile blastula stage—an effect which was carried over into the later stages also.

Although the results tabulated above seemed to be practically conclusive, the question arose as to whether it might not be inter-

esting as well as worth-while to use extracts made up in solvents other than sea water. Experiments made with such extracts should be of use as a check upon the other experiments. Chambers has contended that when a cell is broken into fragments by mechanical means, the small pieces, because of surface tension, usually become spherical in shape, and form a new surface film. Perhaps, then, by breaking down such a possible film by the use of solvents other than water, more of the "formative stuffs" might go into solution.

### *Series 47.*

The extract in this experiment was made by placing the crushed embryos, motile blastulæ, in faintly acidified distilled water. The extract was allowed to stand for twelve hours before it was used. The acidified water was made by adding four drops of glacial acetic acid to 100 c.c. of distilled water.

The controls were, of course, made by adding equal quantities of the acidified water to the sea water when certain quantities of extract were used in the cultures.

TABLE VII.

| Culture. | Amount of Sea Water. | Amount of Extract. | Kind of Extract. | Temp. of Sea Water. | pH of Sea Water. |
|---|---|---|---|---|---|
| *A*..... | 25 c.c. | 5 c.c. | Gastrula | 19.6° C. | 8.2 |
| *A'*..... | 25 c.c. sea water<br>5 c.c. acid water | — | | " | " |
| *B*..... | 25 c.c. | 3 c.c. | " | " | " |
| *B'*..... | 25 c.c. sea water<br>3 c.c. acid water | — | | " | " |
| *C*..... | 25 c.c. | 1 c.c. | " | " | " |
| *C'*..... | 25 c.c. sea water<br>1 c.c. acid water | — | | " | " |
| *D*..... | 50 c.c. | 5 c.c. | " | " | " |
| *D'*..... | 50 c.c. sea water<br>5 c.c. acid water | — | | " | " |

*Later Development and Fate.*—Development in the cultures and controls was very nearly parallel until the blastula stage was reached. At this point, however, a very decided retardation in all the cultures containing extract was noted.

*C* was only very slightly retarded, however, at the blastula stage, and reached the pluteus stage very shortly after *C'*.

*B* and *A* showed many extreme modifications of form. Only a few plutei developed. *A'* and *B'* showed plutei better developed. In all the other cultures with and without extract, plutei were found, although many variations from the normal type were noted.

A separate control of eggs in sea water was kept. This showed a general retardation of development in all the cultures and controls containing even a small per cent. of acid.

*Conclusion.*—In the cultures containing extract an added retardation was shown when comparison with the controls was made. This retardation occurred chiefly at the early non-motile blastula stage.

*Series 59.*

In this experiment the extract was made of ciliated blastulæ in a solution of ether and water (1 part of ether to 2 parts of water, distilled). The water was used merely because it is very difficult to wash the extract from the sand by ether. Since water and ether are only very slightly miscible, the extract had to be shaken very vigorously before it was used.

TABLE VIII.

| Culture. | Amount of Sea Water. | Amount of Extract. | Kind of Extract. | Temp. of Sea Water. | pH of Sea Water. |
|---|---|---|---|---|---|
| *A*..... | 50 c.c. | 5 c.c. | Ciliated blastula | 19.7° C. | 8.2 |
| *A'*..... | 50 c.c. sea water | | | " | " |
| | 5 c.c. of ether-water mixture | — | | | |
| *B*..... | 50 c.c. | 2 c.c. | " | " | " |
| *B'*..... | 50 c.c. sea water | | | " | " |
| | 2 c.c. of ether-water mixture | — | | | |
| *C*..... | 50 c.c. | 1 c.c. | " | " | " |
| *C'*..... | 50 c.c. sea water | | | " | " |
| | 1 c.c. of ether-water mixture | — | | | |

*Later Development and Fate.*—*A* and *A'* showed very irregular cleavage from the first. Development in *B* and *B'*, *C* and *C'* was very nearly parallel through the motile blastula stage, but *A*, *B*, and *C* showed retardation in their development from the blastula stage to the pluteus stage. When the cultures were ex-

amined later, *C'* had many normal plutei; *C* had just a few small plutei, swimming feebly. *B* and *A* were retarded in their development, with fewer and smaller plutei than those in *B'* and *A'*. *A* showed by far the largest number of abnormal forms.

*Conclusion.*—A retardation was noted especially between the blastula and the pluteus stages in the etherial extract cultures.

### *Series 45.*

The extract used in this experiment was made by soaking the embryos (motile blastulæ) after the usual crushing process, in a solution of one part of acetone in one part of distilled water, for 12 hours. This same solution was used in preparing the controls, and was always shaken vigorously before using.

TABLE IX.

| Culture. | Amount of Sea Water. | Amount of Extract. | Kind of Extract. | Temp. of Sea Water. | pH of Sea Water. |
|---|---|---|---|---|---|
| *A*..... | 50 c.c. sea water<br>5 c.c. 50 per cent. acetone | — | | 19.9° C. | 8.2 |
| *A'*..... | 50 c.c. | 5 c.c. | Motile blastula in 50 per cent. acetone | " | " |
| *B*..... | 50 c.c. sea water<br>2 c.c. 50 per cent. acetone | — | | " | " |
| *B'*..... | 50 c.c. | 2 c.c. | " | " | " |
| *C*..... | 50 c.c. sea water<br>1 c.c. 50 per cent. acetone | — | | " | " |
| *C'*..... | 50 c.c. | 1 c.c. | " | " | " |

*Later Development and Fate.*—Development in the cultures and the controls appeared to be very nearly parallel until the early non-motile blastula stage. At that point in development blastulæ, some swimming vigorously, were found in *A* while in *A'* most of the blastulæ were non-motile, although a very few were moving feebly. In *B* and *B'*, *C* and *C'* there was also an evident retardation, although it was not so great as that registered in the *A* and *A'* cultures.

On the following day these readings were made:

*A*—very early plutei; *A'*—a very few late gastrulæ; *B*—early plutei; *B'*—very early plutei; *C* and *C'*—no difference apparent. A few late blastulæ, but mostly early plutei in both.

*Conclusion.*—A definite retardation was noted in the cultures containing extract at the early blastula stage and afterwards.

*Series 53.*

Extracts made up in alcohol ($C_2H_5OH$) must be used in small amounts, or else the alcohol must be a very weak solution, for if the concentration of alcohol in the cultures reaches three or four per cent. so many deaths occur in the blastula stage that comparison with the controls is difficult. Five series of experiments proved unsuccessful on this account.

In the experiment outlined below the extract was made up in 15 per cent. alcohol, and allowed to remain in the tightly corked tube for 24 hours before using.

TABLE XI.

| Culture. | Amount of Sea Water. | Amount of Extract. | Kind of Extract. | Temp. of Sea Water. | pH of Sea Water. |
|---|---|---|---|---|---|
| *A*..... | 50 c.c. | 10 c.c. | Blastula | 21.1° C. | 8.2 |
| *A'*..... | 50 c.c. | 10 c.c. of 15 per cent. alcohol | — | " | " |
| *B*..... | 50 c.c. | 3 c.c. | Blastula | " | " |
| *B'*..... | 50 c.c. | 3 c.c. of 15 per cent. alcohol | — | " | " |
| *C*..... | 50 c.c. | 1.5 c.c. | Blastula | " | " |
| *C'*..... | 50 c.c. | 1.5 c.c. of 15 per cent. alcohol | — | " | " |

*Later Development and Fate.*—Very irregular cleavage in *A* and *A'*. No apparent difference between *B* and *B'*, *C* and *C'* at the blastula stage.

Later: *C'*—many normal plutei.

*C*—a few normal plutei.

*B* and *B'*—*B* very slightly retarded, with fewer and smaller plutei.

*A* and *A'*—as *B* and *B'*, with greater retardation in *A* than in *B*.

*Conclusion.*—Since alcohol, even in very weak concentrations, has such a powerful effect on the developing eggs, no very definite conclusion can be drawn from this series of experiments.

*Series 63.*

In the table below are given some observations made in sea water whose OH concentration had been raised by the addition of 0.8 c.c. *N*/10 NaOH to 50 c.c. sea water. This experiment was also designed as a check upon the experiments in which normal sea water was used. Loeb ('98) brought out the fact that by allowing eggs, fertilized normally, to develop up to the blastula stage, and then dividing the eggs into three lots, to one of which NaOH is added in the proportion of 1.76 c.c. *N*/10 NaOH to 100 c.c. of sea water, a second to which the same amount of *N*/10 HCl is added, and the third for the control, at a given time the bowl containing NaOH shows complete plutei, the HCl culture shows late gastrulæ with a few short-armed plutei, while the control contains many plutei in various stages, and some late gastrulæ. In the report of this experiment Loeb attributed this result to an increased rate of oxidation produced by the OH-ion.[17] Now, on this hypothesis, if the retardation in the development of the eggs due to the presence of the egg extract is a slowing down of the oxidation rate, then presumably the NaOH should neutralize, or antagonize this effect, and we should expect a rate of development more nearly normal.

The experiments proved that just the reverse is true, however, for the effect of the NaOH was not to neutralize, but rather to add to the effect of the extract, since it can be seen by glancing at the table that, if anything, in the culture containing NaOH plus extract, there was a slight retardation in the early cleavage rates compared with the NaOH control. A more marked degree of retardation was shown in the development from the blastula to the pluteus.

This result is in entire agreement with the later work of Loeb.[18] In his book he says that although the rate of development of *Arbacia* can be retarded by the addition of acids to the sea water, he has not succeeded in showing that the rate of development in *Arbacia* eggs can be accelerated by the use of hydroxyl-ions in the sea water. Glaser found that this latter

[17] Glaser, Otto, "Qual. Analysis of Egg Secretions and Extracts of *Arbacia* and *Asterias*," Biol. Bull., Vol. XXVI., No. 6, June, 1914.

[18] Loeb, Jacques, "Artificial Parthenogenesis and Fertilization," Chicago, 1913.

statement seemed to hold good for the early cleavages, since there was no definite increase in rate observed there, but that the rate of development from blastula to pluteus was accelerated. It would appear, however, that from Loeb's account of his experiments, the acceleration mentioned in the first paper was not observed until after the first day, so that there is after all no disagreement between the results obtained by these two investigators.

TABLE XII.

| Culture. | Amount of Sea Water. | Amt. of Extract. | Amount of N/10 NaOH. | Kind of Extract. | Temp. of Sea Water. | pH of Sea Water. | Cleavage Rate. | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 2-cell Stage. | 4-cell Stage. | 8-cell Stage. |
| *A* . . . | 50 c.c. | 3 c.c. | 1.75 c.c. | Blastula | 21.9° C. | 8.2 | 57 | 70 | 76 |
| *A′* . . | 53 c.c. | — | 1.75 c.c. | | " | " | 48 | 54 | 71 |
| *A″* . . | 54.75 c.c. | — | — | | " | " | 40 | 58 | 70 |
| *B* . . . | 50 c.c. | 1.5 c.c. | 1.75 c.c. | " | " | " | 53 | 64 | 72 |
| *B′* . . | 51.5 c.c. | — | 1.75 c.c. | | " | " | 50 | 55 | 69 |
| *B″* . . | 53.25 c.c. | — | — | | " | " | 43 | 53 | 68 |
| *C* . . . | 50 c.c. | 0.5 c.c. | 1.75 c.c. | " | " | " | 49 | 54 | 67 |
| *C′* . . . | 50.5 c.c. | — | 1.75 c.c. | | " | " | 51 | 57 | 64 |
| *C″* . . | 52.25 c.c. | — | — | | " | " | 45 | 51 | 66 |

*Note.*—The time recorded under the cleavage rate is the number of minutes between insemination and the first appearance of the stages indicated.

*Later Development and Fate.*—It is evident that there is only a very slight retardation, if any, in the cultures containing the extract in small amounts during the early cleavages.

At a later reading *A* was found to contain non-motile blastulæ, *A′* motile blastulæ. In *B* and *B′*, *C* and *C′* no considerable difference was observed, except for the fact that in *B′* the blastulæ appeared to be swimming more vigorously than in *B*.

Still later, the reading showed the late gastrulæ in *A* dead while in *A′* were found some plutei of varying abnormality.

*B*—a few plutei, varying somewhat from the normal.

*B′*—flourishing plutei, varying somewhat from the normal.

*C*—as *B*; *C′* as *B′*.

The controls in sea water showed more rapid and normal development at all stages.

*Conclusion.*—A very slight retardation was produced by the use of the extract in the early cleavage stages. This effect was more evident from the blastula to the pluteus.

Experiments were also made using glycerine and chloroform for solvents, but since chloroform appeared to be very difficult to use, and since glycerine even in very small concentrations caused a high degree of mortality in the cultures, no further work was done at this point.

## Analysis and Discussion of Results Obtained.

The end result of the investigations described in this report is that the larvæ of *Arbacia punctulata,* and probably of *Asterias forbesii,* contain substances which, when extracted in sea water or other solvents, *and when present in sufficient concentration,* retard the development of the eggs of the same species.

This retardation was shown in most cases only in a slight degree in the early cleavages, but more markedly in the later stages, especially at the early non-motile blastula stage. There would appear, then, to be certain periods at which the egg is more susceptible to the action of the extracts than at other times.

It is interesting to note in this connection that Lyon ('02) showed that eggs placed in a weak KCN solution gradually lose their resistance to the KCN as development proceeds. At the same time, there appeared to be certain periods at which the eggs were more susceptible to the poison than at other periods.

Child studied the effects of various chemical agents upon the egg of the sea-urchin to prove the existence of axial metabolic gradients as fundamental factors in the development of this form. While it was not at all proposed to attempt in these investigations a corroboration of Child's work, results in the controls in which the agents used as solvents for the larval extracts were the same as those which Child used, were similar, and many of the abnormal types which he described were noted.

Child[19] showed that the effect of KCN, $C_2H_5OH$, $NH_4OH$, NaOH, HCl, and $CH_3COOH$ was a differential inhibition manifesting itself in various deviations from the normal course of

[18a] Lyon, E. P., "Effects of Potassium Cyanide and of Lack of Oxygen Upon the Fertilized Eggs and the Embryos of the Sea-urchin (*Arbacia punctulata*)." *Am. Journ. Physiol.* Vol. VII., pp. 56–75, 1902.

[19] Child, "Larval Development in the Sea Urchin," *Journ. of Morph.,* Vol. XXVIII., 1916–17.

development, as, for example, the variations of the angle of divergence between the arms, the approach of the lateral parts to the median line, and in extreme cases fusion, and many differences in the proportions of the larvæ.

Child also endeavored to show that differential acclimation and recovery may take place, resulting in wide-angled plutei, and an increase in the size of the oral lobe with over-development of the anterior and median regions as compared with the posterior and lateral regions. No specificity in the different form changes produced by the different agents used was noted.

In the experiments described here, also, the deviations from the normal form of the embryos produced by the extracts were so varied that there seems to be no basis for claiming any specificity of action. All the evidence points not toward a qualitative, but a quantitative action of the extract as far as the normal processes of growth and development are involved.

Although it is impossible at this point to state whether or not the different extracts all produce the same effect on the developing *Arbacia* egg, the fact which is to be emphasized is that, however they act, or whatever processes of the egg are chiefly involved, the general effect of the extracts when present in sufficient concentrations is to retard or inhibit the fundamental metabolic processes in some way.

There is a definite normal rate of development for the eggs of each animal, yet this rate may be changed by various conditions of environment. The two most common modifying causes are a change in oxidation (which may be due to a variety of causes) and a change in temperature. Among others, Stockard[20] has shown recently in many experiments that a very wide range in the decrease of developmental rate is very easily brought about by even a slight change in the surrounding temperature or a reduction in the oxygen supply. He has also contended that a normal continuous development may be modified into a discontinuous one by stopping its course during a very early stage.

Now many variations from the normal were found to be produced by the use of the extracts in certain concentrations. Some

[20] Stockard, C. R., "Developmental Rate and Structural Expression," *Amer. Journ. of Anat.*, Vol. XXVIII., No. 2, January 15, 1921.

of these were slight: others were more evident, as, for example, the exogastrulæ, noted in Table II. Their appearance may indicate an arrest of development at the gastrula stage or earlier; in fact, it was noticed that in most cases only after the larvæ had remained for a day at the gastrula stage, did the exogastrulæ appear. Some exogastrulæ, however, appeared after a slowing up of development from the blastula into the gastrula. The further records of experiments planned may render a more definite position on this point possible.

As far as temperature was concerned in carrying out these experiments, it would seem that the comparatively small variation from day to day noted above in the discussion of methods, would scarcely be sufficient to cause the development of abnormal types. And furthermore, if by any chance slight changes in the temperature from day to day might have resulted in the development of abnormalities, these abnormalities should be present in the controls in as great numbers as in the extract cultures. Such was not the case in the investigations outlined in this report.

In considering the part which the extract may have played in altering the normal oxidations in the developing eggs, three possibilities are evident: the rate of oxidation may be increased—in which case it would seem more probable that there would be an acceleration rather than a retardation in the rate of development, or the rate of oxidation may be decreased by the action of substances in the extract upon the eggs, or yet again the rate of oxidation may be decreased simply because the extract itself contains substances which use up the oxygen in the water, and so deprive the eggs of their otherwise available supply. No facts can be brought forward at this time, however, to make any one, or two, or all of these possibilities appear to be the probable explanation of the phenomena under discussion.

It should be emphasized at this point that the retardation caused by the use of the extract results in arrests of development and the production of abnormal types very similar to those caused by the various chemical agents used, although it is not possible of course to state definitely that these result from disturbances of the same metabolic processes.

Another fact which should be emphasized is that there was very evidently a shorter length of life in cultures containing extract than in the controls. This lowered degree of vitality, or of resistance, often manifested itself in a slower rate of movement, particularly at the blastula stage. A possible explanation of this effect may be found in part in the increased danger of bacterial infection which the use of extract affords. The presence of the crushed, and disorganized protoplasm of the extract affords a splendid opportunity for the breeding of bacteria. Thus it must be remembered that in considering the causes of the retardation, and the malformations resulting from the use of the extract, the matter of bacterial infection and more especially the effects of the toxins produced by these bacteria should receive due emphasis. It should be stated, however, that the experiments in which boiled extract was used furnish some evidence, very slight though it may be, that something other than bacterial action is involved in the retardation and the malformation resulting from the use of the extracts.

## Summary.

1. Extracts of *Arbacia* larvæ in the 128–256-cell stage, in the early and in the late blastula, gastrula, and pluteus stages, when present in a sufficiently high concentration, definitely retard the development of eggs of the same species.

If these extracts are used in very low concentrations, the retardation may well lie within the limits of experimental and observational error.

2. The retardation noted is manifested slightly in the early cleavage rates, and more markedly in the later stages of development.

3. Besides retarding development, these embryological extracts often cause cytolysis, arrests of development, and a very noticeable failure of the eggs to develop beyond the early non-motile blastula stage.

4. The very evident tendency of the eggs to stop at the blastula stage suggests that possibly this stage is a stage peculiarly susceptible to the extract and characterized by a general lack of resistance.

5. It is possible that extracts made from larvæ in a certain stage differ qualitatively from those made of larvæ of an earlier or a later stage, but we do not know that this is true, nor do we know that the retardation is associated with any modification of the rate of enzyme action, or permeability, or oxidation, or any specific process. Probably it depends upon the great complexity of the protoplasmic system, and the fact that no one part of that system may be altered considerably without disturbing the equilibrium of that whole system.

6. It is not possible to say at this point that the retardation caused by the extract affects the same metabolic processes as do the KCN, $CH_3COOH$, NaOH, etc., but the use of the extract results in arrests and retardations of development such as are apt to be caused by these chemical agents, and the various types of malformations resulting are often similar.

7. This work does not indicate, then, either the presence or the absence of formative stuffs, but shows that under the conditions given they do not appear. Two possibilities present themselves. If formative stuffs exist, they would seem to be unable to pass into solution in sea water, in lipoid-soluble substances, and in solvents which are able to dissolve carbohydrates. Therefore, if present, they appear to be complex in character and in close association with the protein molecule. The other possibility is that these hypothetical substances may go into solution in certain solvents, but yet may not be able to register any effect upon the developing eggs, because the cell walls of the latter may not be permeable to the formative stuffs in solution.

[1] Conklin, E. G., "Heredity and Environment," p. 185.

[2] Child and Bellamy, "Physiological Isolation by Low Temperature," *Bot. Gaz.*, Vol. LXX., 1920, p. 249–267.

[3] Carnegie Institution of Washington Year Book 1918, p. 55, D. T. MacDougall. Report from the Department of Botanical Research, p. 55.

[4] MacBride, "Textbook of Embryology," Vol. I., p. 526–528.

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[6] Wilson, E. B., "Experimental Studies on Germinal Localization," *Journ. Exp. Zoölogy*, Vol. I., p. 197–269.

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[11] Glaser, Otto, "A Qualitative Analysis of the Egg Secretions and Extracts of *Arbacia* and *Asterias*," BIOL. BULL., Vol. XXVI., No. 6, June, 1914.

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[13] Chambers, Robert, "Personal Communication."

[14] Glaser, Otto, "Qualitative Analysis of Egg Extracts and Secretions of *Arbacia* and *Asterias*," BIOL. BULL., XXVI., 1914.

[15] Fuchs, H. M., "The Action of Egg Secretions on the Fertilizing Power of Sperm," *Arch. für Ent. der Org.*, Vol. XL., 1914. p. 248.

[16] Conklin, E. G., "The Effects of Centrifugal Force on the Eggs of *Crepidula*," *Journ. Exp. Zoölogy*, Vol. XXII., No. 2, February, 1917.

# DIGESTIVE ACTIVITY OF MESENCHYME AND ITS DERIVATIVES.

## II. Proteins as Object (A. Edestin.)

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### 1. Statement of the Problem.

The recently obtained results regarding the digestive activity of the adult splenic cells of the fowl upon two mammalian tumors, the Ehrlich sarcoma and the Crocker Fund tumor 180, have raised a series of problems. Owing to the complexity of the conditions involved in the experiments referred to, they could not be easily attacked and solved at that time.

Has the embryonic mesenchyme, found to be powerless against the proliferation of the two heterogenous tumors in the fowl, no power at all to digest foreign proteins in dead or living form? Is only the adult mesenchyme of the spleen to be regarded as a tissue endowed with a specific digestive function, or can any mesenchymal cell and possibly some of its derivatives, wherever found in the organism, exhibit under definite conditions one of the most fundamental powers of living matter, *i.e.*, that of digesting particulate proteins? Finally, the act of digestion performed by a group of cells, in a well-defined region of the organism, is it to be regarded as a purely local phenomenon, or as a process, the effects of which extend beyond the boundaries of the tissue directly involved?

These questions can be answered only in a fragmentary and insufficient way on the basis of already known data. Embryonic mesenchymal cells were occasionally observed to exercise a phagocytic and digestive action upon dead or weakened cells, but little is known as to whether they possess a similar power against foreign protein. Other than splenic mesenchymal cells

have occasionally been observed in the adult to succeed in completely digesting heterogenous proteins, as for example, catgut of mammalian origin which is digested by the stroma cells of different animals. Though suggestive of a widespread digestive power of the mesenchyme, these observations do not allow any definite conclusions regarding its extent and its various aspects. Finally, the question has hardly been raised, as to whether a digestive activity temporarily developed by the mesenchyme or by its derivatives in a definite region, might not have a general effect upon the whole organism of the animal under experiment. In this connection, the production of induced immunity against tumors by introduction into the organism of particles of tissue of the same species as that bearing the tumor and the immunization against pathogenic bacteria by introducing the same organisms in a weakened state, is of interest. As to how a local process displayed in a well-defined region of the organism can influence its general properties and through what mechanism this can be effected, only few hypotheses and even less data can be found.

In order to approach the problems stated above, different heterogenous, non-injurious proteins should be introduced among stroma cells other than those of the spleen. The study of their digestion, especially when excessive amounts are introduced, would throw light upon the question as to whether easily mobilizable cells from remote parts of the organism will participate in this process along with the cells found in close proximity to the injected masses. The study of the digestion of the substances introduced should be pursued not only until its completion, but special care should be given to the fate of the phagocytes, and a method should be devised for identifying them in any part of the organism which they might enter, either in a passive way or by their own active movements. Rather small animals only can be used, the size of which would not offer insurmountable difficulties for the study of the final distribution of these phagocytes.

A favorable object for such studies has been found in the tadpole. Various amounts of different heterogenous proteins, insoluble in saline, such as edestin, fibrin, coagulated egg albumin, were introduced into the transparent tail. When it was found that

enormous amounts of introduced material were easily digested within the tadpole tail, the details of this process and especially the structure and origin of those cells which exercised this activity were determined. When it was found that the region into which the material was introduced and which was promptly invaded after the experiment by numberless cells was as promptly abandoned by most of them, an attempt was made to determine their new whereabouts and to identify them. This part of the work has so far been only partly successful. The ultimate distribution of the phagocytes will be studied further in greater detail and the results given in another paper.

*Material and Method Used.*

The transparent tail of the tadpole has been frequently used not only as a convenient object for observing normal processes of growth and differentiation, but also as a medium in which the activity of the various stroma cells, under experimental conditions, could be studied with remarkable ease. Work on the tadpole tail has been done in this country chiefly by Eliot R. Clark and Eleanor Linton Clark. They studied in it the growth of vessels in vivo. They introduced under its epidermis microscopic particles of paraffin, India ink, croton oil and starch granules and studied the reaction called forth in the adjacent tissues by the presence of these foreign materials. Some of the materials used by them (paraffin and India ink) were of such a nature as would lead us to expect only a physical reaction from the adjacent cells. Among the other material used by them, the croton oil produced upon the adjacent tissue injurious effects such as it would upon any other living tissue. Uncooked starch granules produced no other effect than foreign bodies, while starch granules cooked to the point of gelatinization proved to be a powerful chemotactic agent for leucocytes. Ingestion of such starch granules took place, but digestion of it was not followed.

The result of this work did not permit of any decisive conclusions regarding the digestive activity of the tadpole stroma for the simple reason that most of the substances used are not digestible. If the same material were introduced into our regu-

lar digestive tract, no other results would be obtained, than those described by the authors in relation to the stroma cells of the tadpole. Paraffin would produce no direct effect upon the lining of the digestive tube, some of the granules of India ink might be incorporated by cells, if a sufficiently long contact could have been secured. Croton oil would produce an intensive inlammation, aseptic if applied by itself in a milieu free of bacteria. Starch granules would be digested, but even these only in certain parts of the digestive tract.

Our experiments consist of introducing various amounts of suspensions of different protein substances into the thin edges of the tadpole tail. The injections were made with extremely fine glass pipettes. After numerous more or less unsuccessful attempts to use rather complex and apparently well-devised methods of injecting, recourse was taken to the simple and efficacious way used by Doctor Clark and courteously demonstrated by him. The substances, suspended in saline solution, were blown into the tissue by lung pressure. The injections were made both into the dorsal and ventral edges of the tail. The epidermis of the tail is easily pierced in the caudal part of its edge by the fine end of a glass pipette containing a thick suspension of a fine pulverized protein, between its dorsal border and the axial strand of the denser tissue. The pipette is then pushed at about equal distance from dorsal and ventral borders of the transparent plate for about 2–4 mm. in the cranial direction. The other end of the pipette is connected with a rubber tube. The substance is introduced into the tissue of the tadpole by blowing into the tube, considerable effort being required in order to force the substance through the glass capillary. It is essential, while blowing, to gradually withdraw the glass pipette from under the skin.

Since large amounts, as well as a few particles of various substances were introduced, medium and large-sized tadpoles were chosen for the experiment. A successful injection could be easily determined, the material injected appearing under the skin as an opaque streak. Though in some cases the substances were introduced in excessive amounts (the strand of injected substance appearing to be over 3 mm. in length and to occupy half

of the thickness of the fin) the animals did not show uneasiness from the effect of the operation. Hemorrhages could not be avoided during injection, but those specimens in which hemorrhage could be macroscopically discerned were eliminated. Observations in vivo were occasionally made and a large number of fixed tadpoles at different stages after injection were secured. The animals were allowed to live from 2 hours to 3 or 4 weeks after the injection.

The first set of experiments, of which a report is given in the present paper, consisted in injecting various amounts of edestin. This substance, insoluble in water, appears macroscopically in the form of minute particles, irregular in shape, rather uniform and rarely exceeding in size the eosinophilic granules of leucocytes. Every edestin granule ingested by cells can easily be identified in an eozin-azur preparation after Zenker-formol fixation, the granules staining a brilliant red.

We wish to express to Professor Gies our great indebtedness for chemically purifying this substance.

*Structure of the Dorsal Fin of a Tadpole Tail.*—A short description of the structure of the edge of the tadpole tail is necessary in order to know what kind of cells will be in immediate contact with the substance introduced into it. It consists of a plate of loose mesenchyme covered on both its surfaces by epidermis. The epidermis contains three differently organized layers of cells, all of them exhibiting frequent mitoses. The superficial layer of cuboidal epithelial cells is covered by a thin cuticular border. The basal layer of epithelial cells consists of characteristic cuboidal and columnar cells containing sharply defined filaments. The middle layer of the epidermis consists of pigmented cells with peculiarly incurved nuclei. Chromatophores in the form of many branched cells are not infrequently found in this layer. The epidermis is lined by a heavy basal membrane. Numerous white fibers traverse the tissue between the two basal membranes.

The loose tissue of the plate contains scattered mesenchymal cells and vessels, a few strands of smooth muscle tissue and nerves. No wandering cells of any kind are normally present in this

tissue. A continuous layer of mesenchymal cells is situated directly under the basal membrane of the epidermis. These cells, as well as those situated within the plate itself, are typical loose connective tissue cells with numerous long cytoplasmic processes, with oval nuclei usually containing well-defined nucleoli and minute particles of chromatin. Occasional mitoses are found among them. Chromatophores are few in this tissue, but are not infrequently found surrounding the vessels and accompanying even tiny vascular branches.

### *Results of the Experiments.*

In describing and analyzing the various phases of the reaction developed around the injected material, a distinction has to be made between the processes due to the injury proper and those which develop in consequence of the presence of foreign matter, digestible particulate protein in this case. The reaction due to the injury is exhibited partly by local cells, partly by elements brought in by the blood current from remote parts of the organism. The reaction due to the injury is, in these experiments, only slight in both of these aspects and is greatly overshadowed, shortly after the injection, by processes depending upon the presence of the injected material.

*Effect Produced by the Injury during Injection.*—Besides a slight injury of the epidermis, a few cells of which are sometimes carried into the mesenchymal plate, the direct consequence of the injection is the boring of a canal in the midst of the loose mesenchymal tissue and the filling of it by finely pulverized proteinic matter. It is remarkable how seldom appreciable hemorrhages are produced, and only very few blood corpuscles are regularly found free in the intercellular spaces as a result of the puncture of a few small vessels. By pushing the glass capillary under the epidermis the tissue is simply compressed, this compression being further exercised by the thick suspension of edestin after the withdrawal of the glass capillary. Microscopically the injected material appears in the form of a rather sharply defined strand of densely aggregated particles. The immediately adjacent tissue becomes slightly edematous and the white fibers traversing the fin plate

swollen. Only few mesenchymal cells are found in direct contact with the edestin particles fifteen minutes after the injection, but even at that time these cells, if compared with those situated at a distance from the injected material, seem to be larger, their processes shorter and plumper and their cytoplasm often filled with tiny vacuoles giving it a foamy appearance. There is no doubt but that these changes in the mesenchymal cells, observed almost immediately after the injection, are not to be regarded as a specific response to the introduction of the edestin. Practically the same changes are seen after an injury produced by the introduction of the glass pipette without injection.

A breaking off of the syncytial arrangement of the cells and formation of typical wandering cells can be observed in those few mesenchymal cells which are in close proximity to the injured tissue. The number of wandering cells formed in loco at the expense of the mesenchymal cells is small indeed, the mesenchymal cells themselves being scarce. These local changes are slow and are quickly overshadowed by the appearance of cellular elements brought in by the blood stream.

*Effect Produced by the Presence of the Injected Substance.*—The changes observed in the region in which the injected masses are situated and dependent upon their presence are in part identical to those found, if only injury with the glass pipette were produced and the injection omitted. They differ greatly, however, in their intensity and in their duration. In describing the changes observed around and within the injected mass, three different phases will be reported under separate sections. (1) Appearance of wandering cells. (2) Digestive processes. (3) Phagocytes after digestion.

1. *Appearance of Wandering Cells.*—As mentioned above, vessels are seldom ruptured during injection and only a few groups of extravasated blood cells are found around the injected material. Among those the white blood corpuscles are seen to exhibit an intensive activity, the erythrocytes, however, lie inert in the intercellular spaces at first. Shortly after the injection the white blood corpuscles become greatly increased in numbers around the injected masses. Not only in nearest proximity but also at a cer-

tain distance there appear a great number of ameboid cells. The granular leucocytes are the earliest to come and during the first hours after injection they are found to be the most numerous. Their structure allows of an easy identification, their polymorphic nucleus being especially characteristic. Most of the leucocytes belong to the neutrophil class. Present in large numbers within the vessels, they are first to emigrate and to appear around the injected masses. Within the vessels and in close proximity to them they present the usual structure, but soon undergo considerable changes in their appearance. Their nuclei are often drawn lengthwise and appear in a thread-like shape. The chromatin in the nucleus is also arranged in the form of rod-like particles. This is of course due to intensive streaming movements exhibited by these cells. But a more substantial change takes place in their cytoplasm. This greatly increases in volume and though still granular is much less distinctly so.

In addition to the granular leucocytes, a number of ameboid cells with round nuclei appear soon. Twelve to fourteen hours after the injection they greatly increase in number and after twenty-four hours they are by far the most numerous among the cells infiltrating the region. These ameboid cells, which become most active in the process of digestion of the injected protein, exhibit the structure characteristic of the small lymphocytes. It is only after a thorough investigation that this conclusion has been reached. A few hours after injection when numerous polymorphonuclear leucocytes have already emigrated, the vessels throughout the tadpole and especially those in proximity to the injection begin to show an increasing number of small lymphocytes. The characteristic structure, which enables an easy recognition of these cells within the vessels, is also present even to minute details in cells which have recently emigrated and are situated near the injected substance. Small in size, they have a round nucleus containing numerous well-defined chromatin particles and no nucleoli. The nuclear membrane is chromatic and sharply defined. The cytoplasm is slightly basophilic and appears in the form of a narrow rim. Outside the vessels they exhibit numerous ameboid processes.

As these cells advance toward the injected mass they exhibit a series of changes, which make them rapidly acquire an entirely different structure and unroll a digestive activity little expected from the small lymphocytes. A rapid transformation of a small lymphocyte into a histiotopic wandering cell thereby takes place. Analogous changes have been observed in small lymphocytes, while in grafts of adult splenic tissue, the small lymphocytes wander out from the grafted tissue into the intercellular spaces of the allantois. Both nucleus and cytoplasm of these cells increase in size. The cytoplasm does not become more basophilic, as usually is the case with the hypertrophying mesenchymal cells. It stains light blue with Azur—II., and is often pervaded with minute vacuoles. The nucleus grows also; its chromatin, especially in the early stages, appears as in small lymphocytes in the form of irregular, rather heavy particles, but is soon converted into tiny fragments. The cells now differ entirely from the small lymphocytes in morphological appearance and wherever found in contact with edestin granules, rapidly ingest them.

The small lymphocytes are scarce in the circulation of a normal tadpole and the appearance of a great number of these cells, not only around, but in later stages within the injected mass, is at first glance rather difficult to account for. A study of the organs of the tadpole at this stage has revealed that the chief hemopoietic center is situated in the kidney. The blood-forming processes here exhibit a peculiarity not yet described in any hemopoietic organs of other embryos. The young blood stem cells, in the form of the lymphoid hemoblasts (large cell, basophilic cytoplasm, clear, round nucleus with a well-formed nucleolus) are here extremely scarce. Lymphoid cells are present in great numbers, but they exhibit the structure of small lymphocytes. The reciprocal relations of the blood cells in the kidney of the tadpole cannot be discussed at present, but it may be pointed out that the same characteristic feature was observed by one of us in the case of the axolotl years ago while studying the hemopoiesis in the perihepatic tissue in this animal.

The vessels in the kidney of the tadpole under experiment are always found to contain numerous small lymphocytes and an

intensive proliferation of these cells is observed in the hemopoietic tissue of this organ. No decrease of small lymphocytes is brought about by the excessive withdrawal of these elements, but, on the contrary, the lymphatic elements seem to become even more numerous and mitoses particularly frequent.

The shifting of numerous small lymphocytes to the injected region produces evidently a stimulating effect upon the hemopoietic tissue of the kidney, similarly to a bleeding of the animal. In this case however the white blood corpuscles only are carried away in greater numbers and those remaining proliferate more intensively. A proliferation of small lymphocytes in the lymphatic tissue has been found under various conditions, after administration of small doses of X-ray in particular. A direct stimulating effect upon the lymphatic tissue was attributed to the action of the X-rays. It is, however, questionable whether this stimulating effect might not prove to be a secondary phenomenon due either to destruction or to an intensive shifting of the small lymphocytes from the region of their origin.

A third category of wandering cells may be recognized around the injected material. They are mobilized mesenchymal cells, in the early stages very few in number and never numerous. They are large, have a rather basophilic cytoplasm, and their nuclei frequently exhibit nucleoli. They have been already mentioned and their first appearance was ascribed rather to the direct effect of the injury than to the presence of injected masses. At the end of the first day after injection a curious activity is observable in the mesenchymal cells which form under the basal membrane a practically uninterrupted layer. These cells, normally flattened against the surface of the basal membrane and in sections appearing fusiform in shape, are now seen to separate from the basal membrane and to protrude their processes in the direction of the injected material. They leave their original places and are found among other wandering cells. Easily recognizable at first they gradually undergo a series of changes in the same direction as described for the small lymphocytes, and at a later stage these cells are no longer recognizable as such.

In order to make a complete picture of the wandering cells in the region of the injection, eosinophilic leucocytes should be men-

tioned, but they are very scarce. An endothelial origin of some of the wandering cells cannot be excluded altogether since occasionally small vessels are injured and their endothelial cells might be transformed into wandering cells. Their number, however, must be of small account indeed in comparison with those brought in by the blood stream.

2. *Digestion of Injected Edestin.*—The process of digestion is inseparably connected in our mind with a specialized system, all of which is derived from the entoderm. This includes a special cavity, in which the process is completed outside of cells and tissues. A group of specifically differentiated secreting organs are furnishing the enzymes, which, though of utmost importance in digestion, still escape a more precise definition of their chemical structure. We still judge of the presence of enzymes by the result produced by them. Owing to the observation of results obtained in the unicellular organism, we easily accord to the protozoa an intracellular power of digestion. In this case the digestive ferments become active within a vacuole surrounded by living cytoplasm, and while the conditions within this vacuole bring about digestion of the ingested food (whether living or dead) they do not affect in the same manner the cytoplasm of the acting organism.

It was Metchnikoff's merit to have pointed out in the multicellular organism the rôle of phagocytosis with subsequent digestion of the ingested material. The phagocytes described by him belonged to two classes distinctly and differently organized, the polymorphonuclear leucocytes and the macrophages. The polymorphonuclear leucocytes did not require much interpretation, but the macrophages became an object of numerous investigations. Their nature and origin are much disputed, possibly because results obtained by study of definite cases and true, if limited to these, were extended beyond the sphere of their control. The macrophages are derived in turn from mesenchymal and from endothelial cells. Blood stem cells (lymphoid hemoblasts) were also seen to become macrophages. A special line of resting or histiotopic wandering cells, eminently phagocytic, was observed to develop from the mesenchyme in later embryonic stages. The

"cellules rhagiocrines" of Renaut and the clasmatocytes of Ranvier are endowed with phagocytic power. The polyblasts of Maximoff, derived from the small lymphocytes, are very active in inflammation processes.

No efforts have been spared to find structural peculiarities characterizing the mononuclear macrophages to the exclusion of lymphocytes. An ingenious method of identifying the macrophages was devised by H. Evans and elaborated by M. Simpson by staining a characteristic set of granules numerous in the mononuclear cells and very scarce in the lymphocytes.

That the class of macrophages, whether or not the mononuclear leucocyte of the blood stream belongs to it, are well-defined structural units, is not a disputed fact, nor is their phagocytic activity doubted. Their origin in the venous sinuses of the spleen, lymph nodes and bone marrow, in the region where endothelium and mesenchyme gradually merge into each other has been admitted. But do these well-established facts make the existence of a much more extended digestive activity in mesenchyme and its derivatives incredible in a multicellular organism? And why should digestive activity be necessarily limited to macrophages of serous cavities and to other cells identical with them as to their structure and origin?

A study of the conditions as they develop gradually in the embryonic organism will allow of an easy realization of the fact, that digestive activity is an inherent attribute of every cell, and that it is retained by that part of the mesoderm which remains the least differentiated in the form of mesenchyme. True that in vertebrates the ectodermal and mesodermal layers are soon separated from the yolk by the entoderm which remains in contact with the yolk and develops into a series of highly specialized digestive organs. But it is equally true that in earlier stages of development the cells of the primitive streak are in direct contact with the yolk and that the cytoplasm of all of them invariably contain yolk granules, which gradually disappear by intracellular digestion. All of the mesodermic phagocytes would derive their digestive power from the cells of the primitive streak, being their direct descendents. Actively manifested by the cells of the meso-

dermal anlage, the intracellular digestive power seems to be lost by a great number of highly differentiated mesodermic structures but is being retained by those mesenchymal elements, which, scattered through the whole organism, remain practically undifferentiated.

The conditions are by far more striking in those classes of animals whose eggs, as in the case of the tadpole, are rich in yolk, but segment completely. Though segmentation results in this case in uneven distribution of yolk among the various groups of cells, nevertheless all of them contain nutritive material and gradually digest it. The mesodermal cells are in this case developed in intimate association with the primitive entoderm, at a time part of it. The primitive blood cells are loaded with yolk granules gradually digested and used up. Later, however, the mesenchyme and its derivatives not being in contact with undigested protein, digestive capacity is not exercised by these tissues. But phagocytic digestive power remains inherent in them and is revealed every time mesenchyme or its derivatives are in the presence of particulate protein.

As seen from the preceding section, the injected mass is surrounded, six hours after injection, by a great number of wandering cells (Fig. 1) of different nature. Some of them are actually seen within the injected mass. The polymorphonuclear leucocytes are not only first to appear around the injected edestin, but they also are the first to bore their way into the rather compact mass of edestin. Wherever a group of edestin particles has been carried by the injection deeper into the tissues and found detached from the more compact mass, the granular leucocytes gather around them and often at this early stage edestin particles are found already ingested by them. While actively ingesting large amounts of edestin particles, the granular leucocytes may attain a considerable size. Their cytoplasm is often seen to be reduced to a narrow rim holding a large amount of ingested material. Its granular structure, becoming less distinctive, while the cell is moving toward the injected mass, has now practically disappeared altogether. But the nucleus retains its polymorphous structure and this makes the identification of these cells always easy. It is remarkable that a great number of polymorphonuclear leucocytes,

found in the midst of the injected mass, are manifesting evident signs of degeneration. A chromatolysis is frequently observed in their nuclei at an early stage and later on these cells, even while containing in their cytoplasm edestin granules, are themselves subject to phagocytosis by other cells.

By far the most important rôle in the actual digestion of the injected mass belongs to those cells which are brought in by the blood stream in the form of small lymphocytes. We do not mean to say that the small lymphocytes as such devour a great amount of edestin and digest it. But there is no doubt that cells, the structure of which is identical to that of the small lymphocytes, emigrate from the blood vessels, approach the injected mass and, while approaching it, promptly change their morphological features. Such cells, lymphoid phagocytes as we will call them, if in contact with the injected mass of edestin, are seen, at the stage of six hours after injection, to contain edestin particles in their cytoplasm. The surface of their cytoplasm becomes indented and tiny cytoplasmic processes are seen to protrude between the injected edestin particles, to entirely surround them, to incorporate them into larger vacuoles and to repeat the same process again and again. Sometimes larger groups of edestin particles are seen to be surrounded simultaneously by larger cytoplasmic processes and incorporated into a common vacuole.

A greater and greater number of cells invade the edestin mass until a stage is reached, about 24 hours after injection, in which all of the edestin granules are found within the cells. At this time (Fig. 2) the conditions within the injected mass are difficult to analyze. The finest celloidin sections give rather obscure pictures. All the edestin granules are found in larger or smaller spaces surrounded by cytoplasmic strands, but the boundaries of the individual cells are indistinct. Cytoplasm, a little more abundant around the nucleus and easily recognizable as a part of a definite cell, is seen to merge gradually into cytoplasmic strands of neighboring cells. The whole region appears in the form of vast plasmodia, here and there interrupted by more or less definite fissures (Fig. 2).

Figure 3 shows the development of these conditions in the peripheral part of the injected mass. A vessel containing ery-

throcytes and a number of small lymphocytes is found to be in close apposition to it. A group of small lymphocytes in its proximity are seen with large ameboid processes. At this stage most of the edestin granules are incorporated in the cytoplasm of the lymphoid phagocytes, only a few groups still remaining outside. Most of them are situated in larger or smaller vacuoles. Apparently one cell may possess a number of such vacuoles. Though in general the cells appear to be well separated from one another, there may also be observed groups of cells which seem to have flown together.

As early as 16–24 hours after injection a change takes place in the numerical relation between the polymorphonuclear leucocytes and the lymphoid phagocytes, now both appearing in the form of large cells with vast vacuoles containing large amounts of edestin granules. The polymorphonuclear leucocytes, even more abundant than the lymphoid phagocytes early after the injection become now scarce and rather difficult to identify. A granular leucocyte, as admitted, is a highly specialized cell, stable and no longer capable of developmental changes. Would such a cell under experimental conditions be still capable of modifying its metabolism so as to manifest a fundamental change in its structure? This idea, incompatible as it seems with our knowledge of histogenesis of blood cells, is forced upon one's mind by the rapidity of the change in the numerical relation of these two different kinds of phagocytes. And only a detailed analysis of the conditions obtaining within the injected mass permits of a final refutation of this idea. The polymorphonuclear leucocytes numerous in early stages become less and less so, because of the association of various factors all working in the same direction. Soon after the first wave of emigration brings around the injected mass a large group of polymorphonuclear leucocytes, lymphocytic cells become more and more abundant within the blood stream and are seen to emigrate in larger and larger numbers. At the same time the polymorphonuclear leucocytes, well within the edestin mass, begin to degenerate in great numbers. In later stages many of them are seen to be ingested by the lymphoid phagocytes. As

a result of the simultaneous association of these conditions, 1 or 2 days after injection almost all of the cells in the area of injection are represented by lymphoid phagocytes.

There is no doubt that while the process of ingesting the edestin takes place, actual digestion begins at about the same time. The digestion in this case is intracellular and similar to that observed in the protozoa. The edestin particles, found in rather large vacuoles, are gradually disappearing, and, on the basis of this observation, the conclusion must be reached, that enzymes are liberated by these cells into vacuoles in conditions permitting of digestion of the protein particles and not affecting the cytoplasm surrounding the vacuole. A rather interesting detail is observed in the digestive activity of the phagocyte. The edestin granules are frequently seen to be surrounded individually by thin strands of cytoplasm. Early after injection, the phagocytes are invariably seen to contain large groups of edestin granules in single vacuoles, but in later stages these groups are seen to be gradually separated by cytoplasmic strands (Fig. 4, four days) until every granule is surrounded separately. Whether, however, this step is necessary is difficult to tell.

While ingestion of edestin by the phagocytes has resulted in the formation of vast indivisible plasmodia (Fig. 2), the digestion and resorption of the edestin leads again to a definition of separate cell units. Two days after injection the region appears very similar to the condition found in the periphery of the injected mass at an early stage (Fig. 3). The phagocytes appear again individually separated and in denser regions only a few plasmodia are observed. This separation is completed 3 or 4 days after injection, as Fig. 4 shows. At this time (4 days after injection) the digestion in most of the cells has been completed. Some of them still show a large vacuole containing a number of edestin granules, but such cells have become scarcer. Other phagocytes contain two or three small vacuoles with a limited number or even with single granules. Cells are observed, also, which do not contain more than 2 or 3 small granules and finally a number of cells are found without any. Digestion in these cells has been apparently completed.

The digestive power is being exercised by the lymphoid phagocytes in a most conspicuous way. It is questionable, however, whether or not polymorphonuclear leucocytes actually digest the edestin particles found rather numerous in their cytoplasm at earlier stages. Mention was made above regarding degenerative changes frequently observed in the leucocytes within the injected mass. But even those leucocytes which persist at a stage when a great part of the edestin has been digested do not seem to prosper. They are now frequently seen ingested by the lymphoid phagocytes and undergoing a digestion within them. In Fig. 4 three lymphoid phagocytes are seen to contain polymorphonuclear leucocytes in their cytoplasm; the latter, themselves, had acted as phagocytes, for numerous edestin granules are still present in their cytoplasm. They succumb, however, now to the phagocytic activity of lymphoid phagocytes.

The structure of the lymphoid phagocytes is so characteristic as to permit an unmistakable judgment regarding their past activity and origin. And the new phase of their digestive activity which now is directed against the polymorphonuclear leucocytes is of great interest. Both lymphoid phagocytes and polymorphonuclear leucocytes develop in the same hemopoietic center and from the same stem cells. Both were brought in by the blood current to the region of the injection; they both moved into the injected mass and began their active ingestion of the edestin particles. But while ingestion by lymphoid phagocytes is followed by an intensive digestive activity, the polymorphonuclear leucocytes, though containing ingested edestin, seem to remain inert. The exercise of digestive activity by the lymphoid phagocytes seem to have stimulated and sharpened their digestive power and they begin to display it against cells of their own kind, the granular leucocytes.

The digestion of the injected mass of edestin is completed in about 6 or 7 days. There is no more trace of edestin granules in the tadpole tail at this time and the whole region, in which the edestin appeared as a compact strand of a 0.3–0.5 mm. in thickness, has been reduced to a line hardly perceptible to the naked eye and no more than 100 $\mu$ thick. Not only has the whole mass

of injected edestin disappeared from this region, but the millions of cells which after 2 days of injection invaded it have left it. There is no indication whatever of degenerative processes in the lymphoid phagocytes. Their changes and fate will be discussed in the following paragraph.

3. *Phagocytes after Digestion.*—A cursory glance and comparison of a small lymphocyte traversing within the blood current the region of injection (Figs. 4 and 5) with the phagocytes scattered through it, is sufficient to establish the difference between these two types of cells, as such. Nevertheless, as seen from the preceding sections, the phagocytes did develop from the small lymphocytes of the blood stream through a series of changes which the emigrated cells exhibited while approaching the injected mass. The exercise of digestive activity by the lymphoid phagocytes seems to have further fixed the acquired structural characters and most of the phagocytes are seen to retain their new structure. They now appear in the form of large cells with especially abundant cytoplasm. They are very similar to the usual types of phagocytes encountered in the loose connective tissue and described under many different names (resting or histiotopic wandering cells, clasmatocytes, macrophages, cellules rhagiocrines). Only the loose mesenchyme in the tadpole does not normally contain any wandering cells. The mesenchymal cells though observed to transform occasionally into wandering cells after injection, are extremely scarce. No mononuclear leucocytes are present in the blood current at that time. The only cells capable of further progressive development and differentiation in the tadpole blood are cells which exhibit the structure of small lymphocytes. They are seen indeed to give rise suddenly to numerous generations of phagocytes. These cells after digestion retain their newly acquired structure and are easily recognizable. They exercise their digestive power locally but do not remain in this region indefinitely. They are seen to increase in number while supplies of injected edestin last, but as soon as all of it is consumed, the immigration of new cells stops. The phagocytes remain in this region while they are actively digesting the injected edestin, but then begin to emigrate individually. By far the great-

est part of the phagocytes are seen to leave the region and settle in new places.

The phagocytes are seen to be very active in leaving the region of the injection as soon as they have completed the digestion of the ingested particles. The large aggregations of these cells are being dispersed, and 4 days after injection the egress of phagocytes is very intensive. At this time lymphoid phagocytes can be detected at a good distance from their previous location. Small groups of phagocytes are gathered directly under the epidermis. Two or three weeks after injection they can still be recognized as cell units with definite structure. What is even more remarkable is, that the cells are seen not infrequently to undergo mitosis.

Though on the basis of the present experiments a complete description of the distribution of the phagocytes cannot be given, there are undoubtful indications that their new whereabouts are not confined to the tadpole tail. The phagocytes, while moving from the region of injection, come not infrequently across vessels. Vessels have grown by this time also into the injected region, and numerous phagocytes are now seen to be directly applied against the thin-walled vessels. It is also possible to detect some of these phagocytes within the vessels. It is natural that such pictures are extremely rare, because the phagocytes, once entered into a vessel, are promptly carried away.

The uninterrupted egress of the phagocytes reduces the wide strands of densely accumulated phagocytes to a hardly visible scar (Fig. 5). The cells in it are of two kinds: Typical mesenchymal cells and typical histiotopic or resting wandering cells. The latter are the less numerous. As compared with Figure 4, Figure 5 shows that the egress of the phagocytes is chiefly responsible for the loosening of the tissue and for the shrinking of the previously wide strand of the densely infiltrated region.

## Conclusions.

The introduction of a large amount of edestin into the mesenchymal plate of the tail of the tadpole produces a local and a general reaction in the organism. The local reaction consists in a response of mobile and mobilisable cells and results in a dense infiltration of the injected mass with consecutive ingestion and

digestion of the injected mass. The injected suspension of edestin is a powerful chemotactic agent for both the granular leucocytes and small lymphocytic cells.

Secondarily a general reaction appears in the blood-forming tissue of the kidney. It consists in intensive proliferative processes which seem to be in relation with the egress of small lymphocytes from this tissue.

The lymphocytic cells emigrated from the vessels gather around the injected mass, hypertrophy and gradually transform into typical histiotopic wandering cells or lymphoid phagocytes. They ingest large amounts of edestin granules and exercise a digestive capacity. The digestive activity manifested by these cells seems both to stabilize the newly acquired structure of the phagocytes and to sharpen further their digestive power.

The granular leucocytes though active in ingesting the edestin granules do not seem to be capable of digesting them. These cells, especially those containing edestin particles in their cytoplasm, are seen to succumb finally to the digestive activity of the lymphatic phagocytes. There exist undoubtful indications that some of the phagocytes have derived from the mesenchymal cells, but their rôle cannot be easily determined in these experiments.

Two days after injection all the edestin granules are found within the phagocytes; seven days after injection all of the edestin has disappeared.

The digestive processes within the injected region not only result in the formation of a generation of new eminently phagocytic cells, but seem to reflect secondarily upon the whole organism. Innumerable cells of the type of small lymphocytes are not only transformed into lymphoid phagocytes, but these phagocytes are soon found at a considerable distance from the region of injection and part of them are probably distributed through the blood current to more distant parts of the organism.

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## EXPLANATION OF FIGURES.

All the figures are camera lucida drawings made with the Zeiss apochromat oil immersion 2 mm., and with the compensatory ocular 8.

### PLATE I.

FIG. 1. Zone of injection 6 hours after experiment. Edge of the injected strand of edestin at the right side of the figure. A vessel at the left contains erythrocytes, numerous small lymphocytes and one neutrophil polymorphonuclear leucocyte. The emigrated small lymphocytes in the proximity of the vessel are small. They hypertrophy while approaching the injected edestin. A number of granular leucocytes and three lymphoid phagocytes have penetrated the mass of edestin and begin to ingest its particles.

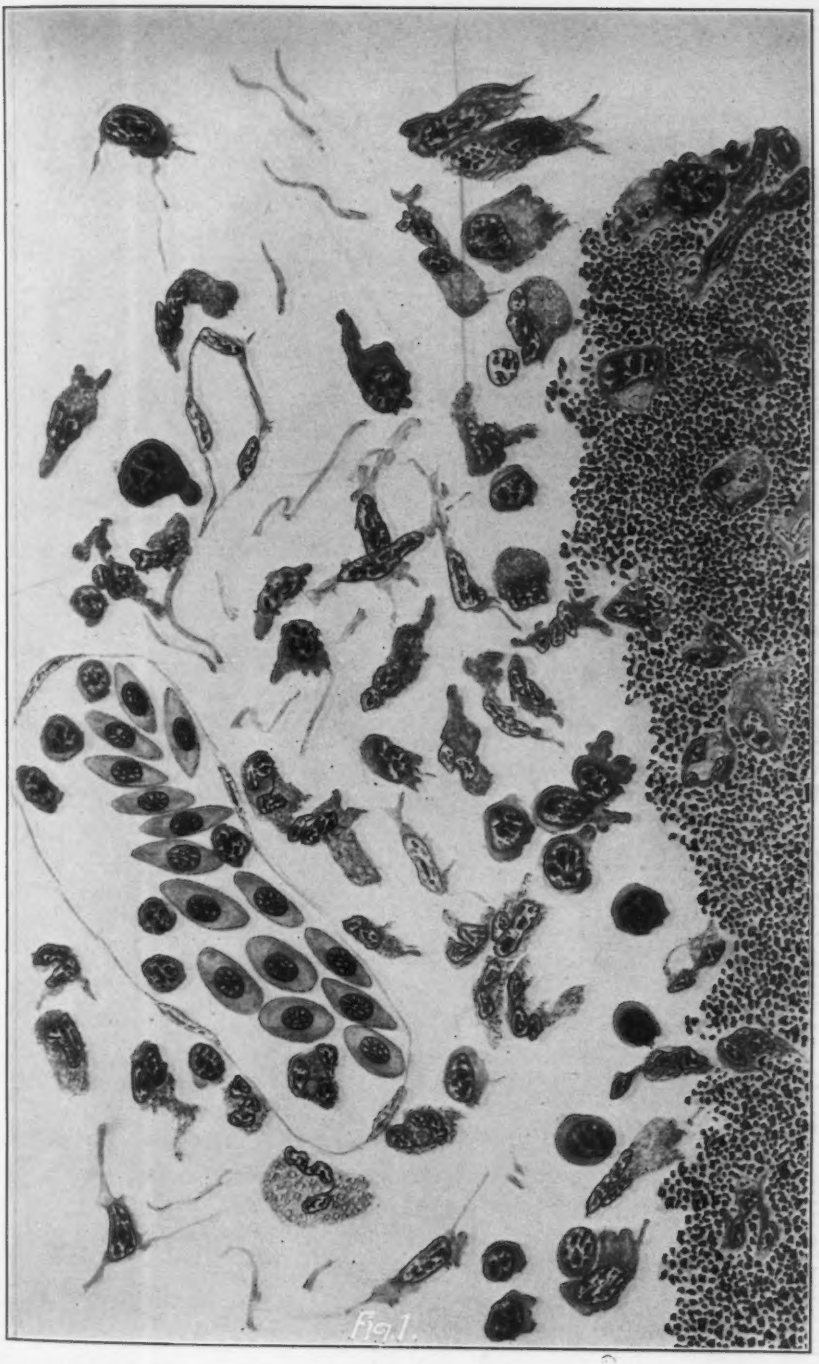


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PLATE II.

FIG. 2. Zone of injection 24 hours after operation. Central part of the large strand of ingested edestin. The cells form a uniform plasmodium. The edestin granules are seen to be separated into groups and surrounded by thin cytoplasmic processes.

FIG. 3. Zone of injection 24 hours after operation. Edge of the large strand of injected edestin. A vessel is seen to contain erythrocytes, small lymphocytes and a granular leucocyte. Most of the phagocytes contain large amounts of edestin and appear as separate cells, though a few of them have flown together.

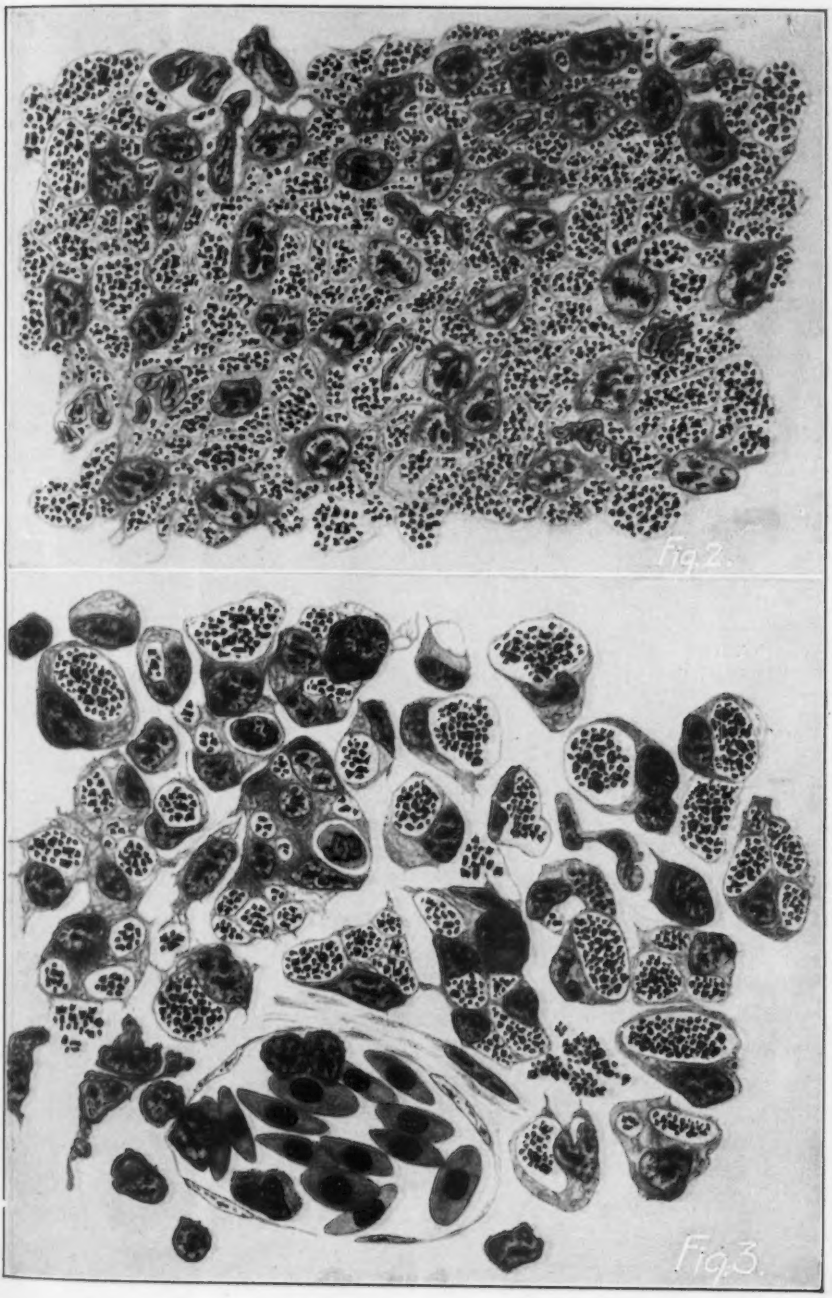


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PLATE III.

FIG. 4. Zone of injection 4 days after experiment. A great number of phagocytes have completed the digestion of the ingested edestin; others contain still either small groups or even single particles of edestin. Three granular leucocytes with edestin in their cytoplasm are seen to have been ingested by the lymphoid phagocytes. The phagocytes become much less numerous than in the preceding stage.

FIG. 5. Zone of injection 7 days after experiment. A thin scar remains after completion of digestion of the injected edestin. It consists of mesenchymal cells, scarce histiotopic wandering cells (lymphoid phagocytes) and a few vessels traversing the zone.

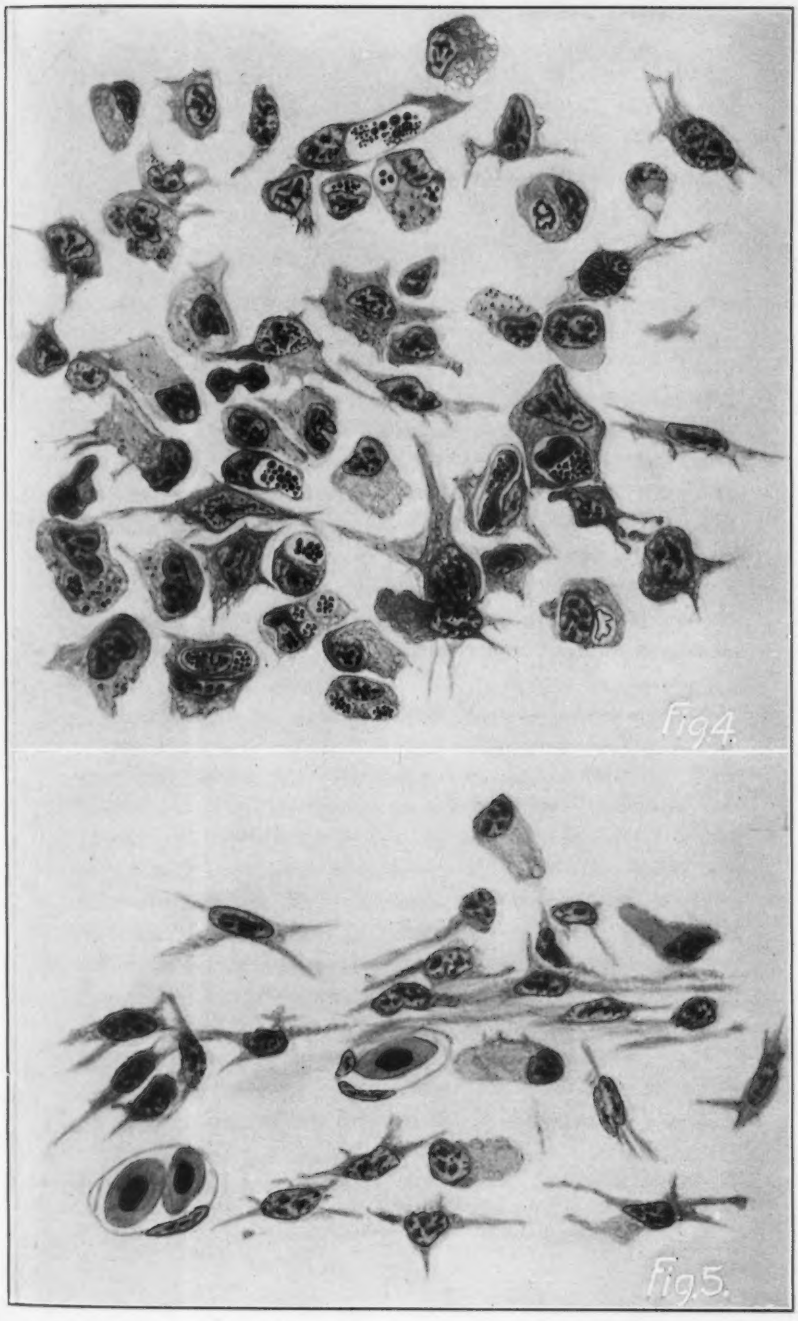
Fig 4.
Fig 5.

# A STUDY OF INFLUENCES WHICH MAY AFFECT THE SEX-RATIO OF THE DEER-MOUSE (*PEROMYSCUS*).

FRANCIS B. SUMNER, MARY E. McDANIEL AND RALPH R. HUESTIS.

## INTRODUCTION.

For the past eight years the senior author has been conducting breeding experiments upon California deer-mice, chiefly subspecies of *Peromyscus maniculatus*.[1] Throughout this period, fairly complete records have been kept of the births, with a view to the ultimate use of these data in a study like the present.[2] The work of tabulating the crude data and of computing the values herein presented has been chiefly performed by the junior authors. The senior author has, however, supervised the work throughout, and assumes responsibility for the accuracy of these various figures.

The data upon which this report is based are not in all respects as complete as might be desired for the study of sex-ratios, though we do not believe that their value is seriously affected by these limitations. Thus the number of individuals in a brood was frequently not determined until the expiration of some days, or even as much as two weeks after birth. As a rule, the brood was discovered on the day of its birth, or within one or two days thereafter. The number of young was commonly recorded at that time, in cases where it was possible to do so without seriously disturbing the mother. At latest, the number was recorded about 16 days after birth, at which time we have regarded it as safe to clean out the cage and change the nesting material.

Record of the number of each sex in a brood was not usually made at the time of this first count. In some cases it was made

[1] Sumner, 1920, and papers therein cited.

[2] There are here included a few hundred mice from the records of Mr. Huestis, and a few hundred others from the records of Mr. H. H. Collins. We are indebted to Mr. Collins for permission to use these last.

within the next few weeks; in others it was deferred until the time of marking, when the broods were broken up, those of different sex being segregated, and each individual being registered and given its proper serial number and identification mark. There has been no uniformity of practice in regard to the age at which the mice have been thus separated, marked and registered. Whenever practicable, this has been done within six or eight weeks after birth, but three months or more have sometimes elapsed before it has been convenient to do so. When, as is commonly true, no deaths have occurred during the interval which has elapsed since the first count, no harm can have resulted from thus deferring the record of sex.

A few words are worth while at this point in reference to the possible bearing which such imperfections in our records might be supposed to have upon the results set forth in this paper. As already stated, we do not believe that they seriously affect their value. In the first place, such errors as actually exist are ones of omission. The number recorded for certain broods is doubtless too small, owing to the occasional presence of stillborn young, or of ones which died within the few days following birth. On the other hand, we are confident that mistakes in the identification of the sexes have been so infrequent as to be negligible.

Regarding this matter of incompleteness of the entries, several things must be said. Firstly, it is doubtful whether any records are possible which are perfectly complete in this respect. It is a practical impossibility to inspect every brood immediately after birth, and in the interval which elapses the mother may eat such stillborn or feeble offspring as are present.

In the second place, the number of dead or defective members of a litter is small in *Peromyscus*, as compared, for example, with ordinary white mice. Stillborn young may be very common among the latter. In our experience they have certainly been rare among the former. As regards very early postnatal mortality, this is, from the nature of the case, difficult even to estimate from the data at our disposal. Reference to the death rate somewhat later in life may, however, be instructive. Of the 1,567 broods, comprising 5,050 young, which are listed in our records,

only 305 broods, or less than 20 per cent., showed any mortality whatever between the time of the first count (16 days or less) and the time of marking (60 ± days). The number of young of unknown sex which died during this interval was 384 or about seven and a half per cent. of the total.

Thus it is not possible that a differential death rate (unless very marked) would seriously affect the sex ratio of the survivors. We have, however, fully considered the possible influence of such a differential death rate, and have endeavored to determine its degree, if actual. This has been done by comparing the sex ratios in broods in which deaths (of undetermined sex) are known to have occurred with broods in which no deaths are known to have occurred (see below). Unfortunately the figures are available for only a very small number of offspring of known sex (40) which died from natural causes before the "sexing" of the broods to which they belonged.

As already stated, the total number of broods recorded is 1,567, comprising 5,050 young, or an average of 3.22 mice per brood. According to sex, these were distributed as follows:

| | |
|---|---|
| Males | 2,295 |
| Females | 2,357 |
| Sex undetermined (dead, killed and escaped) | 398 |
| Total | 5,050 |

For those of known sex the sex ratio (number of males per hundred females) is $97.37 \pm 1.93$.[1]

[1] In computing the probable errors we have employed a formula furnished us by Dr. Raymond Pearl, viz.:

$$\pm 67.45\ (1 + R)\sqrt{\frac{R}{n}},$$

in which $R$ is the number of males divided by the number of females, and $n$ the total number of individuals concerned.

Our colleague Dr. G. F. McEwen has computed a somewhat simpler formula for the sex ratio:

$$\pm .6745\sqrt{\frac{p\,q}{n}} \times 4,$$

in which $p$ and $q$ are the percentages of males and females respectively. This gives approximately the same values as Dr. Pearl's formula when the sex ratios do not depart widely from 100. Neither formula is accurate when the departures are very wide.

It must be stated that the probable errors employed in this paper are about

Since, as will be shown presently, there are rather wide seasonal differences in the sex ratios found by us, and since the different months are represented very unequally in our records, it is of interest to present the mean of these separate monthly ratios. This figure is 95.65.

Both of the foregoing figures are distinctly smaller than have been given by various writers for rats and mice,[1] as well as for man and some other animals.[2] In most cases a decided excess of males has been reported. Miss King (1918), for example, from the records of 2,818 white rats born in her "stock" (*i.e.*, unselected) series, obtained a sex ratio of 104.6.

Some attention should here be devoted to the possibility, already referred to, that the sex ratio which we have obtained for *Peromyscus* has been influenced by differential mortality. As is well known, the sex ratio among stillborn infants is very high, being frequently given as 130 or more;[3] and for cattle a similarly higher prenatal mortality among the males has been reported.[4] For *Peromyscus* we have no data on this subject since the sex of stillborn young was in no case determined. As regards early post-natal mortality, also, our direct evidence is very meager, so much so as to be almost worthless. 424 deaths occurred between the date of counting and the date of marking and registration. Owing to cannibalism and other causes, it was frequently impossible to determine the sex of these dead individuals, and in many other cases we neglected to do so; but this was done in 40 cases. Restricting our consideration to those mice which died during the first two months of life, we have 31 individuals, of which 20 were males and 11 females. From these figures one

twice as great as those which would be obtained by another formula which has been widely followed (see Pearl and Pearl, 1908), and are therefore much safer as a basis for estimates of the significance of results. Indeed it turned out that certain highly interesting conclusions which we had drawn at the outset had to be relinquished on this account. It may be added that the conclusions of certain other writers are greatly weakened if the formula here employed is substituted.

1 King, 1911, 1918.

2 Morgan, 1907, pp. 365–366; 1913, pp. 230–231.

3 Morgan, 1907, p. 368; Schultz, 1918, p. 264.

4 Jewell, 1921.

might be led to suppose that the young males were subject to a mortality about twice as great as the females. But aside from the extremely limited numbers here concerned, there are other reasons for believing that no such marked differential mortality exists in this case.

Separate computations have been made for the broods in which no deaths were recorded and for the broods in which deaths are known to have occurred. Of the former there were 1,301 broods, containing 4,081 individuals, 2,020 being males and 2,061 females. The sex ratio here is $98.01 \pm 2.07$.

Of the broods which are known to have sustained losses prior to marking there were 297, originally comprising 1,079 individuals. The number dying (or missing) was 438, of which 398 were of unknown sex. The surviving 641 mice comprised 312 males and 329 females, giving a sex ratio of $94.83 \pm 5.06$. Thus we do find a slight difference between the complete and the depleted broods, though the difference is a quite non-significant one, statistically speaking. The reasonable inference is that the mortality, during the period here concerned, is approximately equal for the two sexes. In any case, there can be no such disparity in their respective death rates as the meager record of identified dead might lead one to suppose.

Seven possible influences, which have been held by various writers, to affect the sex ratios of animals, have been considered in our treatment of the data at hand. These are: (1) season, (2) size of the litter, (3) race, (4) hybridization, (5) inbreeding and outbreeding, (6) order of birth, (7) diet. As will appear in the ensuing pages, we have some evidence that the first, second and fourth of these influences are actually effective in the case of *Peromyscus*, though perhaps in no case can this evidence be regarded as wholly conclusive. As regards the other four possible influences, the evidence is inconclusive or is quite negative.

To the foregoing list of possible factors affecting the sex ratio we may add an eighth, namely the *year*. Surprising and inexplicable as the fact may be, we have found large and sometimes significant differences between the sex ratios for certain of the years covered by our observations. Indeed these differences are

statistically more certain than any of the others which appear in our records. Such a relationship is not, of course, an ultimate fact, incapable of further analysis. But it does not seem to be dependent upon any of the other agencies for whose influence we have positive evidence, nor are we at present able to offer even a plausible explanation of it.

We shall consider, in turn, the supposed influences which have been enumerated above.

## Season.

Before proceeding to discuss the possible influence of season upon the sex ratio of *Peromyscus*, it should be stated that the mice in question have, with a few exceptions, been born and reared under atmospheric conditions closely approaching those of the outside world. The building ("murarium") used for the purpose was specially constructed with a view to securing this result throughout the year.

Our studies of the relation between season and the sex ratio emphasize the danger of basing conclusions of this sort upon inadequate statistical data, even though the results may at first seem to be "significant," according to accepted standards. Before we included the records for the last two years (1920 and 1921), the evidence of a well-marked biennial rhythm in the sex ratio of *Peromyscus* seemed fairly conclusive. The seasonal cycle, when plotted graphically, was perfectly consistent, there being an uninterrupted rise and fall twice annually. Likewise the differences between the highest and the lowest ratios were of tolerably high "significance." The inclusion of the data for these two later years greatly weakens the evidence for a definite seasonal cycle in the sex ratio. But the possibility still seems to be great enough, and the facts, if true, of sufficient interest, to warrant our presenting the evidence rather briefly.

The following table gives the sex ratio for each month of the year, likewise (in parenthesis) the number of individuals upon which this ratio is based. The table is based upon the combined data for all of the years and for all the different series of mice.

| | | |
|---|---|---|
| January | (185) | 88.78 ± 8.92 |
| February | (287) | 90.07 ± 7.04 |
| March | (722) | 106.29 ± 5.14 |
| April | (343) | 113.04 ± 8.24 |
| May | (506) | 94.62 ± 5.63 |
| June | (405) | 87.50 ± 5.80 |
| July | (347) | 91.71 ± 6.57 |
| August | (565) | 103.97 ± 5.84 |
| September | (445) | 96.04 ± 6.13 |
| October | (406) | 107.14 ± 7.17 |
| November | (236) | 78.79 ± 6.93 |
| December | (205) | 89.81 ± 8.37 |

These figures and the accompanying graph (Fig. 1) reveal the existence of two annual maxima, one occurring in March and April, the other extending from August to October. Alternating with these are summer and winter minima.

It will be seen that the differences between the successive months, taken by themselves, are of very doubtful significance, while even the difference between the highest and lowest months of the year (April and November) is only a little more than three times its probable error. With the exception of September, however, the seasonal cycle presents a perfectly consistent picture, there being an almost uninterrupted rise and fall twice annually.

The dotted line in the figure is based upon the date of conception, instead of the date of birth. The date of conception has been computed, for each brood, by subtracting 22 days from the date of birth, this being the usual period of gestation.[1] Such a procedure has inevitably resulted in the transference of part, though not all of the broods to the month immediately preceding. Were the period of gestation exactly one month, the one graph would be an exact duplication of the other. As it is, they present some obvious differences of form. The chief of these is the division of the fall "maximum" into two entirely distinct peaks.

Since, for various reasons, it does not seem probable that the differences between the consecutive months should be regarded as significant, we may profitably combine our monthly birth records into four seasons of three months each. Reference to the table of monthly ratios shows that we may distinguish two high periods

[1] Sumner, 1916.

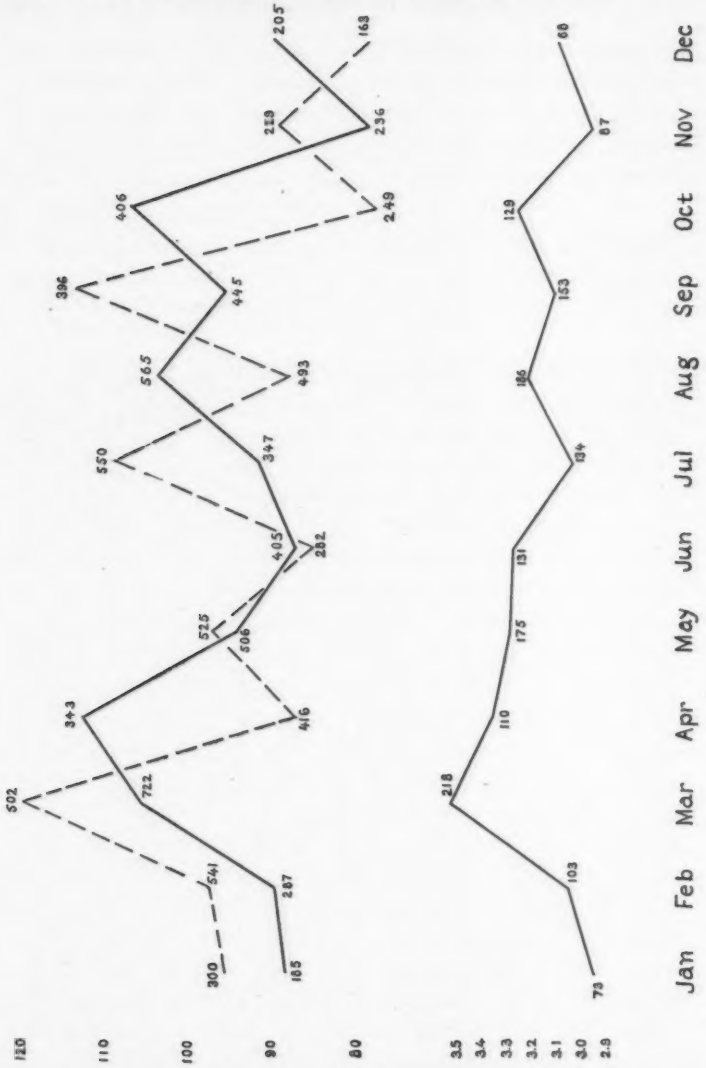


FIG. 1. Above: the sex ratio of *Peromyscus* for each month of the year, computed from all the material. The continuous line is based upon the date of birth, the broken line upon the date of conception. Ordinates indicate sex ratios (number of males per hundred females). The figures along the graphs denote the number of individuals born (or conceived) during each month of the year.

Below: mean size of brood for each month of the year. Figures along line = number of broods.

and two low periods annually. The sex ratios for these four periods are as follows:

| | | |
|---|---|---|
| (1) | February–April | $104.23 \pm 3.85$ |
| (2) | May–July | $91.48 \pm 3.48$ |
| (3) | August–October | $102.29 \pm 3.68$ |
| (4) | November–January | $85.21 \pm 4.62$ |

The greatest difference between two of these ratios is that between the first and fourth periods. This is $19.02 \pm 6.01$. Taken by itself, such a difference is commonly regarded as having a probable significance.

When the same broods are grouped according to date of conception, the figures become:

| | | |
|---|---|---|
| (1) | January to March | $105.35 \pm 3.85$ |
| (2) | April to June | $91.09 \pm 3.50$ |
| (3) | July to September | $102.68 \pm 3.66$ |
| (4) | October to December | $82.10 \pm 4.38$ |

Here, again, the greatest difference is between the first and fourth periods, being, in this case $23.25 \pm 5.83$, or almost exactly four times its probable error.

Figure 2 is based upon the sex ratios for the four 3-month periods of the year, both when the broods are grouped according to date of birth and according to date of conception. The two graphs are seen to be in very close agreement, though the divisions between the 3-month periods have, in the second case, been advanced by one month.

It is when we subdivide our material into groups of one sort or another that the inconstancy of these seasonal differences becomes evident. Dividing our broods according to years is not very instructive, owing to the relatively small number born in any one year, taken singly, and particularly to the exceedingly meager records for certain months. These graphs (not here reproduced) show various extreme fluctuations which must be regarded as purely accidental. Five[1] of the seven "curves" show, however, what may be regarded as spring and fall maxima, though the position of these varies somewhat from year to year. Two of

[1] Those for 1915 to 1919 inclusive, 1920 and 1921 being the exceptions. No graph is possible for the first year (1914) owing to the small number of months represented in our records.

them, on the contrary (including one of the fullest years) give little indication of such relations.

More instructive are the results of combining these years into three groups of two to three years each, and plotting the seasonal cycle from the combined data for each of these groups (Fig. 3). The resulting graphs require no further discussion.

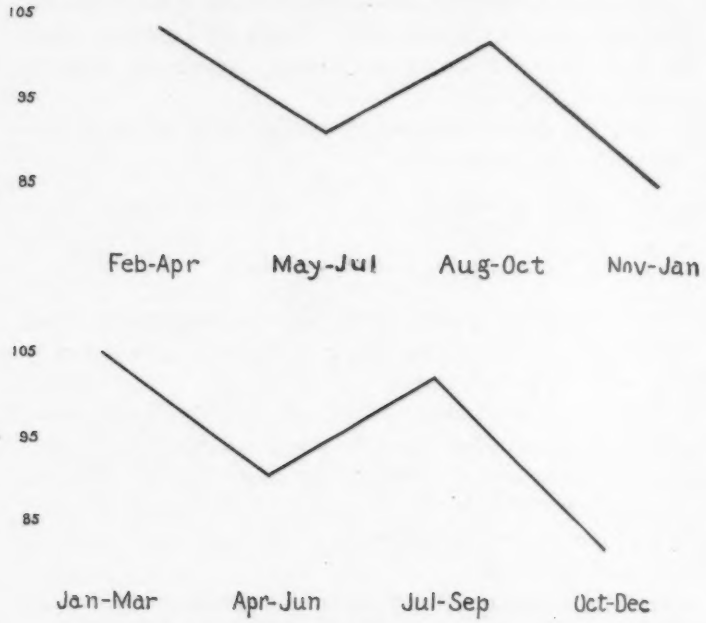


FIG. 2. Sex ratio graphs obtained by combining monthly records into "seasons" of three months each. The lower graph is based upon date of conception, the upper upon date of birth.

The data for the separate years were also grouped into 3-month periods, and graphs plotted for inspection (not reproduced). In 6 cases out of 7, there was a well-marked fall from the first to the second period, just as in the lot as a whole (Fig. 2). In 6 cases out of 7, likewise, there was a rise from the second to the third period. In only 3 cases out of 7, however, was there a fall from the third period to the fourth, one line remaining horizontal and the other three rising. (One of these last is based upon only 19 individuals.)

Two other methods of subdividing our material have been employed, these giving conflicting results. When we plot separately

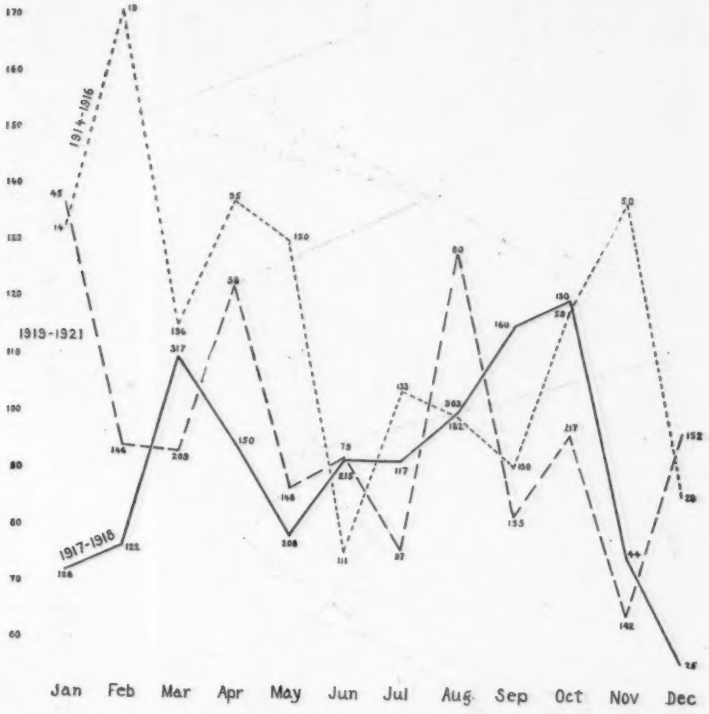


FIG. 3. Seasonal variations in the sex ratio, for three groups of years, treated separately. The continuous line (1917–1918) is based upon the largest number (1,917). Ordinates indicate sex ratios. Numbers along graphs indicate numbers born in each month. Certain of the larger fluctuations will be seen to be due to limited numbers of individuals (*e.g.*, in 1914–1916).

the hybrid mice and those of pure race (Fig. 4), we find that each of these groups displays a pretty well marked biennial rhythm. This is, however, much more pronounced in the former group than in the latter, and it is also to be noted that the "fall maximum" occurs in one case in August, in the other in October. In passing, let us point to the distinctly higher sex ratios shown by the hybrids throughout most of the year, a fact to which we shall refer again.

Our material was likewise subdivided according to the size of the brood, the seasonal cycles for broods of one to six individuals

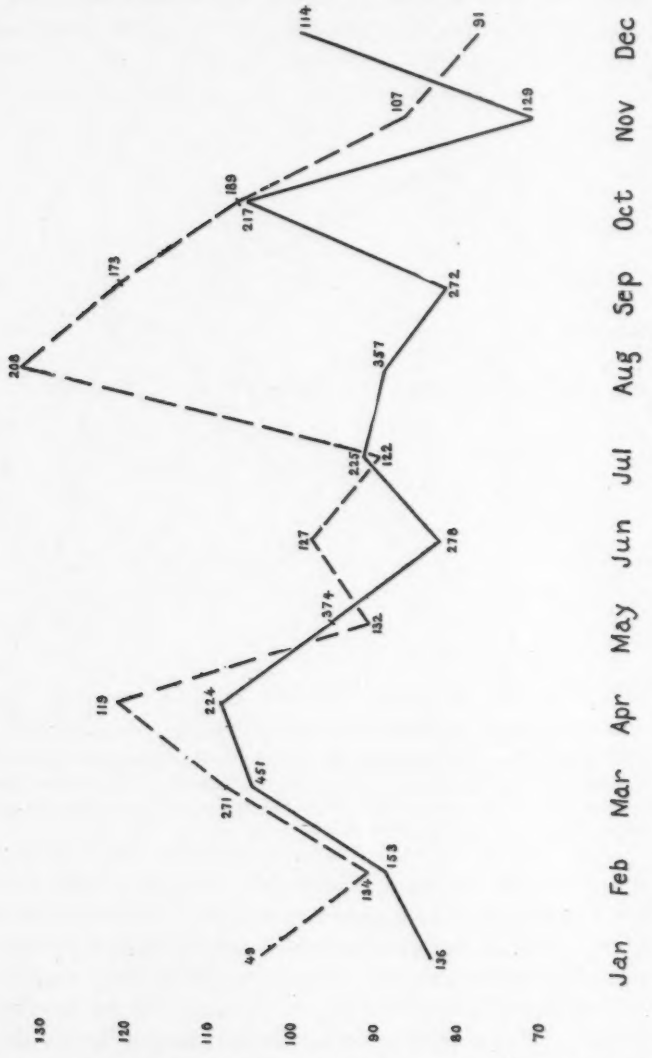


FIG. 4. Seasonal variations in the sex ratio for pure and hybrid stock, treated separately. Continuous line = pure. Broken line = hybrids.

respectively being plotted separately. Here again, the numbers comprised in some of these groups were so small that the graphs

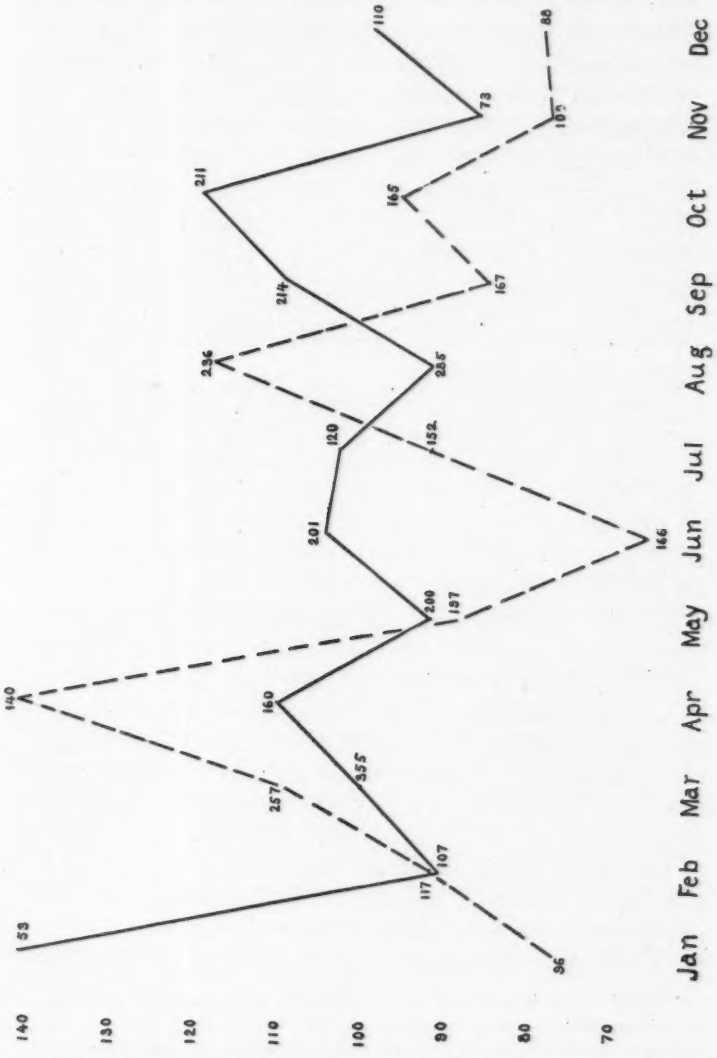


FIG. 5. Seasonal variations in the sex ratio, for small (1 to 3) and large (4 to 9) broods respectively. Continuous line = large. Broken line = small.

as a whole were rather confusing. We have, however, combined the data for small broods (one to three individuals) and for large broods (four to nine individuals) respectively (Fig. 5). This gives us two groups, each comprising about half of our material. The former group exhibits a very pronounced biennial rhythm with maxima in April and August.[1] The curve for the latter group, however, although based on somewhat greater numbers than the first, agrees only in having a well-marked fall maximum (September–October), with lower ratios in the summer and winter. There is but slight evidence of a spring maximum, and there are other irregularities in the "curve" for this large subdivision of our material. In general, none of these differences are of probable statistical significance. The high point in January cannot be regarded seriously, owing to the small number of individuals (53) for that month.

It must be conceded that the differences in the seasonal cycle, shown by these two groups of mice, seriously weaken our evidence for the existence of significant seasonal differences of any sort. For it does not seem likely that mice belonging to broods of different sizes actually behave differently in this respect.[2] It seems more likely that, for present purposes, we have two random samples of the population.

However, the fact stands that considerable differences have been found in the sex ratios of *Peromyscus* at different times of the year, whether or not these differences are due to "chance" (*i.e.*, errors of random sampling). Let us grant, for the moment, that the differences are not accidental. It then remains to consider whether they are due to season *per se*. It will be shown below that our hybrids as a class give considerably higher sex ratios than do the mice of pure strain. Also, quite independently of this last, it will be shown that mice born in different years differ widely in their sex ratios. The question arises: Can it be possible that the seasonal differences which we find are due either to the unequal distribution of hybrid and pure-bred mice through-

[1] The difference between the figures for April and June is more than four times its probable error.

[2] Even this is not impossible, however, for there is some evidence (see below) that smaller and larger broods differ in their mean sex ratios.

out the twelve months, or to the unequal part which the mice of different years may have had in determining the various monthly ratios?

The first of these possibilities is set aside by reference to Figure 4, showing the seasonal cycles of the pure and hybrid stocks plotted separately. The second we have tested by ascertaining the actual effect of each year's quota in determining the sex ratio for each of the twelve months.[1] But an examination of these figures (which it is not worth while to present here) does not support such an explanation of our seasonal cycle. Unless, therefore, all of these monthly differences are due to errors of random sampling, they are probably caused in some more or less direct way, by seasonal changes of temperature or some other physical agent.

The findings of certain other investigators regarding seasonal differences in the sex ratio may be appropriately considered here. King and Stotsenburg (1915) have presented data from 7,619 white rats reared at the Wistar Institute. The exact temperature conditions existing in the animal quarters are not discussed by the authors, though it is stated that the provisions for heat regulation were inadequate, and that the rats suffered greatly from heat during the summer months. It may be presumed that the rooms were heated to some extent during the winter.

When grouped by months, the entire data of King and Stotsenburg show what might be construed as a biennial rhythm. As regards the position of these annual maxima and minima, their results are in some respects in direct contradiction to ours. One well-defined maximum covers the period from June to August, another, the period from October to December. Minima occur in March and September. The second of these is of brief duration, being bounded on either side by months having high sex

[1] A new set of monthly ratios was computed as follows: For each month, the number born in each of our years was multiplied by the general sex ratio for that year, and the sum of these products was divided by the total number born in that month. If the monthly differences which we have discussed above were due to the different seasonal distribution of the mice born in different years, this new set of monthly ratios ought to show much the same relations as the set computed earlier.

ratios. The former, on the other hand, is merely the lowest point in a prolonged period of low sex ratios.

When plotted according to 3-month periods, the records of King and Stotsenburg show an annual minimum in the spring (March to May) and an annual maximum in the summer (June to August), while an intermediate condition is indicated for both fall and winter.[1] Our figures, on the other hand, show maxima in the spring and fall, minima in the summer and winter, though the "seasons" adopted by us commence a month earlier than those adopted by the former authors.

The marked differences found between the seasonal cycles of *Peromyscus* and the white rat might plausibly be attributed either to the difference of species or to differences in the environmental conditions under which the two sets of experiments were conducted. On the other hand, it appears to us that the reality of the seasonal cycle described by King and Stotsenburg is subject to exactly the same doubts as that described by us. The fact that the two groups of years into which the rat experiments were divided gave quite contradictory relations for the winter months certainly gives us reason for such skepticism.[2]

Heape (1907) gives evidence for the existence of seasonal differences in the sex ratios of dogs. The records for nearly 18,000 greyhounds show sex ratios which fluctuate irregularly between 111 and 128 during the months of January to September inclusive. In October, however, the curve rises to 145, in November to 180, and in December to 195. As regards the dates of conception, these last three months become August, September and October. For collies, on the contrary, he says that there is "no evidence that conception at any particular time of year affects the proportion of the sexes born." It must be added, however, that his figures for the collies indicate very considerable monthly differ-

[1] The foregoing statements refer to the entire data of King and Stotsenburg. The material is divided by the authors into two groups which are partially discordant with one another. One of these (that for 1911–1913) gives a graph which is almost exactly the converse of ours.

[2] It is possible, however, that unknown influences (other than random sampling) caused differences in the sex ratio from one year to another. That such annual fluctuations actually occur in *Peromyscus* will be pointed out below.

ences among the sex ratios, and that some of these differences would seem to be of statistical significance.

For man, Heape (1909, 1909*a*) has compiled data, based upon census records of the births of more than 175,000 whites and negroes in Cuba. Unfortunately he does not give the number of births nor the sex ratios, for every month of the three-year period with which he deals, but only the figures for certain selected months of each year, namely, those showing the highest and lowest birth rates. We are therefore obliged to take Heape's conclusions to a large extent upon his own authority.

There are, he tells us (1909*a*) two seasons of high birth rate, a major one in July and August, and a less marked one in November and December. There are likewise two seasons of low birth rate, the chief of these being in January and February, the lesser one in September. During the periods of high birth rate, we are told, the sex ratios are relatively low (102.9 to 105.5 for whites; 93.3 to 104.1 for negroes). During the periods of low birth rate, on the other hand, the sex ratios are relatively high (106.2 to 113.0 for whites; 99.8 to 116.3 for negroes). Thus, there are "two sharply-defined breeding seasons each year . . . experienced by both whites and colored at the same time," one of these being more marked than the other.

These breeding seasons, Heape contends, are not related to the periods of the year at which marriages are most frequent. He seeks for correlations between certain meteorological conditions and the periods at which conceptions occur with greatest and with least frequency. The former periods would naturally fall in October–November and February–March, the latter in April–May and in December. In regard to these seasonal correlations, Heape states: "Reference to records of temperature, barometric pressure, humidity, etc., shows that these bursts of reproductive activity always take place at times when there is a marked change of climate; the one in the autumn . . ., the other in the early months of the year . . . it is obviously not a definite temperature, but the experience of a *change of temperature* which induces this boisterous generative activity" (1909, pp. 35, 36). Likewise, "my tables demonstrate that the greatest excess of females is produced at times of greatest fertility."

King and Stotsenburg likewise believe that there is a relation between breeding activity and the sex ratio. It is stated that "the rat breeds more readily in the spring than in any other season of the year, and there is a second, less pronounced, period of sexual activity in the early fall," while relatively fewer litters are produced in July and August. Their graphs show low sex ratios in the spring, followed by high ones in the summer, and low ones again in the fall. As already stated, the results for winter are contradictory with one another.

Our records for *Peromyscus* are unfortunately not adapted to revealing definite periods of increased or diminished reproductive activity, since the matings were to a large extent controlled in accordance with the demands of the breeding experiments.[1] But it is to be noted that the seasonal fluctuations which we have found in the sex ratio of *Peromyscus* correspond as little with those reported by Heape as they do with those reported by King and Stotsenburg. The relations which Heape believes to be shown by the Cuban statistics, even if applicable to man in that particular locality, cannot be generalized for all mammals nor for all places.

It is worth remarking at this point that the differences just discussed, between our findings and those of certain other writers, are typical of the conflicting results which pervade the entire literature of sex determination.

## Size of the Broods.

The 1,567 broods here recorded ranged in size from 1 to 9, though we have only nine records of broods containing over 6 young (seven of 7, one of 8, and one of 9). The mean size of all these broods was 3.22. This figure naturally varied somewhat according to the year (2.57 to 3.67), the season (2.96 to 3.53), and the race (2.76 to 3.78), etc.

The size of the litter will first be considered in relation to its possible influence upon the sex ratio. This part of our discussion

[1] The numbers born in November, December and January are well below those born in any other months of the year, and it is likely that this fact is due, in part, to an actual slowing down of reproduction, at least under the conditions of our experiments. But it is questionable whether any other seasonal differences in fecundity can be fairly inferred from our records.

need not be long, since the correlation in question is decidedly doubtful. The following table gives the sex ratios for mice belonging to broods containing from one to 7 (+) individuals respectively, excluding broods in which individuals of unknown sex are known to have died, and ones of probably mixed parentage (dual maternity).

| Number in Brood. | Males. | Females. | Ratio. |
|---|---|---|---|
| 1 | 64 | 61 | 104.9 ± 12.7 |
| 2 | 232 | 252 | 92.1 ± 5.6 |
| 3 | 624 | 657 | 95.0 ± 3.6 |
| 4 | 677 | 667 | 101.5 ± 3.7 |
| 5 | 264 | 256 | 103.1 ± 6.1 |
| 6 | 88 | 92 | 95.6 ± 9.6 |
| 7–9 | 28 | 17 | 164.7 ± 33.8 |

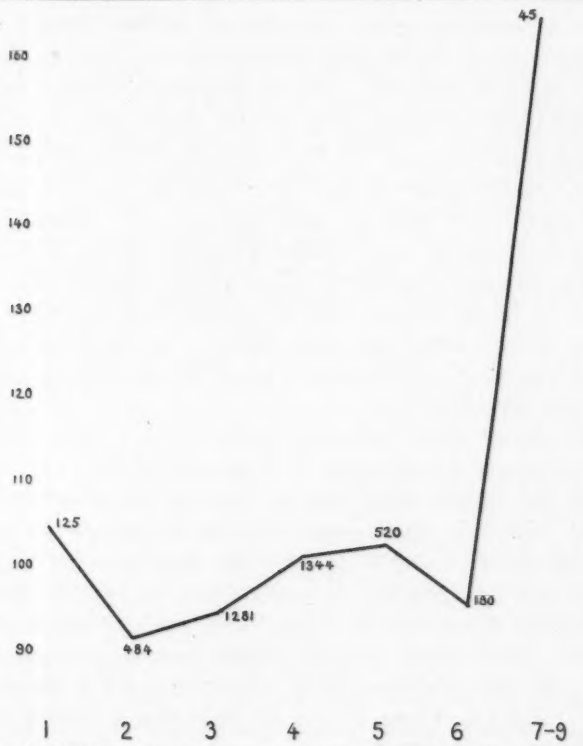


FIG. 6. Variations in the mean sex ratio, according to the size of the brood.

It will be seen (Fig. 6) that the sex ratio for broods containing from 2 to 5 individuals, inclusive, are arranged in a regularly ascending series, while the last group (7 and over) gives us a ratio far in excess of any of the others. Exceptions to this general trend are groups 1 and 6, though it will be noted that these two (as well as the last) are based upon relatively small numbers.

Despite the absolute magnitude of some of these differences, however, their statistical significance is in every case very doubtful. In most instances they are less than twice their own probable errors. When we combine broods of 1 to 3 and 4 to 9, inclusive, the sex ratios of these major size groups become $94.85 \pm 2.94$ and $102.42 \pm 3.01$, respectively. The difference between these values is rather less than twice its probable error. Here, then, as in the case of season, we cannot say with any confidence that the size of the brood is a factor which influences the sex ratio.

It might be inferred, moreover, that this correlation, even if actual, results from the fact that the smaller broods are ones which have been depleted by unrecorded deaths, and that the death rate has here been higher among the males. This explanation derives some support from the fact (p. 128) that among the very small number of identified dead included in our records, the males greatly preponderated. It is rendered improbable, however, by the fact (p. 129) that broods which are known to have been depleted give a sex ratio which is not significantly lower than that shown by the broods in which no deaths are known to have occurred.

Nor do our records afford any ground for the belief that these differences in the sex ratios of broods of different size depend upon the unequal distribution of such broods throughout the year. And it is equally improbable that the seasonal cycle, discussed above, is dependent upon the seasonal distribution of large and small broods. It happens that the seasonal cycle, in respect to the mean size of the brood, does correspond pretty closely, on the whole, with the seasonal cycle for sex ratio (Fig. 1). But the differences, in the former case, are so slight that they could not at most account for more than a fraction of the differences in the latter.

King and Stotsenburg obtained conflicting results from two large subdivisions of their material, when arranged to test the possible correlation between sex ratio and size of litter. The authors cautiously conclude that "the lack of uniformity in the results of this arrangement of data indicate that apparently there is no well-defined relation between litter size and sex in the albino rat."

Weldon (1907), on the other hand, believed that he had obtained some evidence that a positive correlation existed between the size of the litter and the sex ratio in mice. This relation was discernable only when the mean size of litter in different generations was considered, not being evident in respect to individual litters within a generation. The figures presented are not, however, very impressive.

*Combinations of the Sexes in Individual Broods.*—Thus far, the relative numbers of the sexes in broods of different size have been dealt with by methods of mass statistics. The total for each sex has been computed, and the ratio between these totals obtained. Such treatment would entirely obscure one possible phenomenon of high interest, namely the tendency of members of a litter to agree with one another in respect to sex. Do we, for example, encounter broods consisting of four or five of the same sex more frequently than would result from chance?

This question we have endeavored to answer by arranging broods of each size in groups according to the number of each sex present. For example, broods of three present four possible combinations: 3 ♂, 2 ♂ + 1 ♀, 1 ♂ + 2 ♀, 3 ♀. In table A are included only broods in which no deaths are known to have occurred. There are given the actual number of broods containing a given combination of males and females, likewise the "expected" number, to the nearest integer. In computing these last figures, it has been assumed that males and females are equally likely to be produced. The close approximation to equality in the material as a whole seems to warrant this procedure.

When we consider the comparatively small number of broods present in most of these groups, the agreement between the actual and the expected figures is remarkably close. This agreement is

## TABLE A.

NUMBER OF BROODS CONTAINING EACH POSSIBLE COMBINATION OF MALES AND FEMALES.

| Number in brood | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1..... | Combinations..... | | | | | | 1♂ | | 1♀ | | | | | |
| | Number of broods (actual)... | | | | | | 64 | | 61 | | | | | |
| | Expected number..... | | | | | | 62 | | 62 | | | | | |
| 2..... | Combinations..... | | | | | 2♂ | | 1♂, 1♀ | | 2♀ | | | | |
| | Number of broods (actual)... | | | | | 56 | | 120 | | 66 | | | | |
| | Expected number..... | | | | | 60 | | 121 | | 60 | | | | |
| 3..... | Combinations..... | | | | 3♂ | | 2♂, 1♀ | | 1♂, 2♀ | | 3♀ | | | |
| | Number of broods (actual)... | | | | 53 | | 147 | | 171 | | 56 | | | |
| | Expected number..... | | | | 53 | | 160 | | 160 | | 53 | | | |
| 4..... | Combinations..... | | | 4♂ | | 3♂, 1♀ | | 2♂, 2♀ | | 1♂, 3♀ | | 4♀ | | |
| | Number of broods (actual)... | | | 18 | | 88 | | 129 | | 83 | | 18 | | |
| | Expected number..... | | | 21 | | 84 | | 126 | | 84 | | 21 | | |
| 5..... | Combinations..... | | 5♂ | | 4♂, 1♀ | | 3♂, 2♀ | | 2♂, 3♀ | | 1♂, 4♀ | | 5♀ | |
| | Number of broods (actual)... | | 4 | | 22 | | 27 | | 29 | | 17 | | 5 | |
| | Expected number..... | | 3 | | 16 | | 32 | | 32 | | 16 | | 3 | |
| 6..... | Combinations..... | 6♂ | | 5♂, 1♀ | | 4♂, 2♀ | | 3♂, 3♀ | | 2♂, 4♀ | | 1♂, 5♀ | | 6♀ |
| | Number of broods (actual)... | 0 | | 0 | | 11 | | 9 | | 7 | | 3 | | 0 |
| | Expected number..... | 0 | | 3 | | 7 | | 9 | | 7 | | 3 | | 0 |

particularly striking when we compare the actual and expected totals for all of the broods in which all members were of the same sex. The actual number of such homosexual litters, among broods containing from 2 to 6 individuals inclusive, was 276. The most probable number on the assumption of purely random sex-production was 274.[1] Thus, not only do we find approximately equal numbers of males and females in the population as a whole, but in single broods the distribution follows the laws of chance. There is no tendency for fetuses (or germ cells) developing in the same parents at the same time to give rise to organisms of the same sex.

It may seem, on first thought, that such evidence is conclusive against the efficacy of any factor, except the chance meeting of the gametes, which might be supposed to play a part in determining the sex ratio. On the assumption, for example, that seasonal influences of an undetermined character may affect this ratio, should we not expect undue proportions of all-male broods at one time of year, and of all-female broods at another time, and should not this fact result in a preponderance of homosexual broods throughout the year as a whole? We have not computed the proportions of all-male and all-female broods by months, but the probability of the excessive production of such broods at certain times of the year may be granted. We must, however, point out the equal probability of an excessive production of evenly balanced broods at other times of the year. During these months of average sex ratios, we should presumably have not only equal numbers of males and females produced *in the aggregate*, but a tendency toward balanced broods on the part of *individual mothers*. The possibility of sex-determining agencies other than chance combinations of the gametes in fertilization is quite unaffected by these results.

One important conclusion seems justified, however, by this utter absence of any tendency toward the preponderant production of homesexual litters. This is the non-occurrence of polyembryony or true twinning, at least with sufficient frequency to affect the results.

[1] If we include fractions (a more exact procedure) this number becomes 277. Surely this is a close "fit!"

Newcomb (1904), from a study of 7,896 families, concluded that, after making allowance for a slight preponderance of males, the sexes in each family were distributed according to chance. There was no tendency toward an excessive production of families consisting wholly of one sex. A consideration of multiple births, however, based upon French and German statistics, showed the existence of a pronounced tendency toward agreement in sex on the part of twins and triplets. Biologists would have confidently predicted such a situation, owing to the familiar phenomenon of duplicate ("identical") twinning, but Newcomb strangely overlooked this explanation of his results and found in them support for the idea that sex is determined by unknown causes operating during development.

## Race.

In the following table, the mice of "pure" (*i.e.*, non-hybrid) stock[1] are grouped according to the geographic race (subspecies) to which they belong. The La Jolla representatives of the subspecies *gambeli* are here kept distinct from those coming from the more northern localities (Berkeley and Calistoga). This is partly due to the appreciable morphological differences between these local races of *gambeli*, partly to the fact that the northern representatives were nearly all born during the months of May to August, a circumstance which may account in part for the extremely low sex ratio found in this group.

| Subspecies. | Males. | Females. | Ratio. |
|---|---|---|---|
| *gambeli* (Berkeley and Calistoga) | 49 | 72 | 68.06±8.50 |
| *gambeli* (La Jolla) | 770 | 840 | 91.67±3.07 |
| *sonoriensis* | 350 | 373 | 93.83±4.70 |
| *rubidus* | 150 | 124 | 120.97±9.91 |
| Total | 1,319 | 1,409 | 93.61±2.36 |

In making a comparison between these races, we may reasonably leave out of consideration the small group of Berkeley and Calistoga *gambeli*. The two main groups (numerically speaking), *sonoriensis* and La Jolla *gambeli*, differ from one another by an

[1] Except *P. maniculatus dubius* and *P. eremicus*, for which the numbers are too small.

amount which is less than half of its probable error. These two, however, particularly the latter, differ from *rubidus* by amounts which are possibly significant. But it is not certain that these figures imply the existence of actual racial differences in respect to sex ratio. One thing may be stated with confidence, however, namely, that the high ratio shown by *rubidus* is not due to any peculiarity in the seasonal distribution of its births. This has been shown by the following procedure. The sex ratio proper to each month, as computed for the pure races in general, has been weighted by the number of *rubidus* born in the corresponding month, and the product-sum divided by the total number of mice of this subspecies. The mean ratio thus obtained is 92.77, a figure even lower than the ratio for all mice of pure race.

The existence of racial differences in the sex ratio have been pointed out for man. According to Newcomb (1904) and Heape (1909) the ratio is higher for the white race than for negroes. Heape (1907) likewise gives different figures for different races of dogs, these figures ranging from 96 to 136. There is little analogy, perhaps, between the artificial "races" of dogs and the geographic "races" of wild mammals, and the comparison with man is of equally doubtful validity.

## Hybridization.

Of the 4,652 young of known sex, 2,930 belonged to "pure" subspecies, 1,722 to subspecific hybrids. The former were not, of course, pure, in the sense of having been closely inbred for many generations, or of being uniform in genetic composition. Their purity was relative. Among the hybrids are included, not only those of the $F_1$, $F_2$ or later generations, but various backcrosses with the parent stocks as well.[1] This procedure seems warranted, according to any theory of the behavior of subspecific characters in hybridization. There is no clear segregation of the parental stocks in the $F_2$ generation, as would be expected if

[1] Only subspecific crosses are here included. Crosses between "mutant" races within a subspecies, or between one of these and the parent stock, have not been regarded as hybrids for the purposes of this study. Of these, however, the number is not sufficient to materially affect the ratio for the "pure" stock, even though such an effect were known to occur.

these stocks differed by only one or by a few pairs of unit factors. In consequence the $F_2$ and back-cross individuals are in a true sense *hybrids*.

The proportions of the sexes in the two lots are as follows:

| | Males. | Females. | Ratio. |
|---|---|---|---|
| Pure | 1,414 | 1,516 | 93.27 ± 2.32 |
| Hybrids | 881 | 841 | 104.76 ± 3.41 |

The difference between these two ratios is 11.49 ± 4.1, thus being slightly less than three times its probable error.[1] These figures by themselves, therefore, cannot be held to prove at all conclusively the existence of a higher sex ratio among subspecific hybrids in *Peromyscus*. But the probability of such a difference is greatly increased by several circumstances. (1) It has already been shown (Fig. 4) that the "hybrid" ratio exceeds the "pure" in nine months out of twelve, and that in two of the three exceptional cases the differences are trivial. (2) The hybrid ratio was higher in six of the seven years, taken separately (Fig. 7), and the single exception here is based upon a very small number of individuals. (3) The ratios for all of the five separate groups of hybrids are larger than that for the pure stock. (4) Such a difference accords well with the bulk of the evidence for other species of animals.

It is quite unlikely that the difference here found is due to any of the other factors with which the sex ratio of *Peromyscus* has been found to be correlated. As regards season, it has already been pointed out that this difference between the hybrid and pure material holds for nearly all the months of the year. Likewise the difference is very nearly the same when we deal, in each case, with the mean of the monthly sex ratios.

Again, as regards the size of the brood, the mean difference, in this respect, between the hybrid and pure stocks (3.26 and 3.20) is such that it could not exercise an appreciable influence upon the sex ratio.

[1] It may be pointed out that when this probable error is computed according to a method widely used (see p. 127), the difference is more than five times the latter.

Nor can the differences be due to the varying proportions of these two groups which were born in different years. As already mentioned, the difference holds for six of the seven complete years of our records, the exception being unimportant, owing to the small numbers concerned. Likewise a computation analogous to that described on p. 139 shows that distribution by years (on the assumption of an equal sex ratio in hybrid and pure stock) could account for only a small fraction (2.5) of difference actually found.

Owing to the relatively small numbers, it is hardly worth while to discuss at any length the separate subspecific crosses which were made. The figures for the five different groups, with the numbers (in parenthesis) on which they are based are as follows: 114.63 (176), 114.11 (698), 97.27 (361), 95.65 (270) and 93.75 (217). Thus, all of these figures are higher than the ratio for the "pure" stock, though in the last case the difference is trivial, and in only one case is it as much as three times its probable error. Of the smallest of these figures it should be stated, however, that it is based entirely upon mice which were born from May to December, inclusive, thus missing the spring maximum.

All in all, the evidence, if not wholly decisive, points rather strongly to the conclusion that in *Peromyscus* hybridization *per se* results in increasing the proportion of males which are born.

Various previous writers have called attention to the larger proportions of males resulting from hybrid matings. To mention but a few of these cases, Guyer (1909) has presented evidence of this sort for various bird crosses,[1] Riddle (1917) for pigeons, Harrison (1919) for lepidoptera, Pearl and Pearl (1908) and Little (1919) for man, King (1911) for rats and mice. In certain of the cases discussed by Guyer and Harrison[2] the proportion of males reached 100 per cent.

[1] In some of the bird crosses discussed by Phillips (1921) the males do not seem to be in excess, but the number of individuals is small, and the data are not presented with a view to answering this question.

[2] Harrison's results are complicated by the different behavior of various reciprocal crosses in respect to the sex ratio, by the preponderance of females in some cases, and by the appearance of "intersexes," *i.e.*, intermediates between the two sexes.

Miss King gives the data for 425 hybrids ($F_1$, $F_2$ and $F_3$) between the Norway and the albino rat. The sex ratio found was 119.07 or 118.11, depending upon whether the entire number was included, or whether consideration was restricted to the most reliable series (277). In either case, the difference cannot be regarded as significant, according to the standard here adopted. In the first case, it is $14.5 \pm 8.3$.[1] Miss King has also computed the sex ratio, from the records given by von Guaita of hybrids between albino mice and Japanese waltzing mice. The ratio is 113.17, but this is based on only 356 mice of known sex, and is surely of doubtful value.

Pearl and Pearl give the results of computations, based upon vital statistics of the city of Buenos Aires. They have compared the sex ratios resulting from Argentine $\times$ Italian and Argentine $\times$ Spanish matings with those resulting from matings within each "pure" race. The figures for the "hybrid" group were in each case the larger, and the authors believed that the difference was significant in three of the four cases taken singly. But when these probable errors are computed according to the formula now adopted by Dr. Pearl (see p. 127), the significance of these various differences becomes doubtful in all cases except one. That between the sex ratio for the Italian-Argentine cross and for pure Italian stock, is $4.95 \pm 1.05$. In all of the other cases the probable error is half or more times as great as the difference. Despite the great numbers comprised in these statistics, there is thus some doubt as to the reality of these differences. And their biological meaning is further obscured by the fact that the "pure" races differ among themselves in respect to their sex ratios quite as significantly as they differ from the "hybrids";[2] likewise by the fact that the highest "pure" ratio ( the Spanish $\times$ Spanish) is very nearly identical with the lower of the two "hybrid" ratios. It should be added that the authors themselves did not express any great confidence that the higher ratios of the mixed matings are due to hybridization *per se*. We shall discuss later the significant yearly differences which are shown in Table I. of the

[1] Assuming $104.6 \pm 2.77$ as the normal ratio for the white rat.

[2] The same reservation must, though certainly with less cogency, be made in the case of our own data, discussed above.

paper of Pearl and Pearl, though not referred to by these authors.

Little (1919) has reported upon a smaller number of offspring of "pure" and "hybrid" stocks, born at the Sloane Maternity Hospital in New York City. The matings considered were those within or between the following "races": English, Irish, Scotch, Italian, Russian, Greek, Austrian and German. Of the births of "pure" stock, there were 5,753, and these gave a sex ratio of $106.27 \pm 1.81$. Of the "hybrid" births there were 1,305, giving a sex ratio of $121.56 \pm 4.49$. The difference between these ratios is a trifle over three times its probable error. In each case stillbirths were included. It is of interest to note that the proportion of stillbirths among the "hybrid" matings was considerably lower than among the "pure."

If the foregoing figures represent an actual biological difference between the two groups under consideration, the magnitude of this difference is surprising. For each of the "pure" races under consideration is, of course, not a race at all, biologically speaking, but is itself a hybrid mixture. The basis employed in these records was obviouslly nationality, not ethnic stock.

In a later paper (1920) Little has divided his material into "European pure," "European hybrid," "United States white," "British West Indian colored," and "United States colored." In general, the significance of Little's findings is greatly diminished if the probable errors are computed according to the formula which has been employed in the present paper. Thus computed, these are about double the errors given by Little. But at least three of this author's differences remain of probable significance, viz.: those between European pure (104.54) and European hybrid (122.86), between European pure (104.54) and U. S. white (118.33), and between U. S. white (118.33) and U. S. colored (96.12). It is of considerable interest that there is no significant difference between European hybrid and United States white. "This shows," as Little remarks, "that in the data studied, the United States white ratio is essentially that of a hybrid race," *i.e.*, an even more hybrid one than the European "races."

## INBREEDING.

A comparison of "inbred" and "outbred" stock (as defined below) was made at a preliminary stage of these studies, when the total number of individuals dealt with was about 1,700 less than at present. The results of this comparison were so completely negative and the amount of work involved is so great,[1] that it has not been thought worth while to revise them.

For the purpose of comparing the sex ratios, our material was divided into two classes, "outbred" and "inbred." The first class included offspring from matings between unrelated individuals or individuals not related more closely than first cousins; the second included matings between parents and offspring or brothers and sisters.

In the first class, we have 2,346 individuals, comprising 1,171 males and 1,175 females. The sex ratio is $99.66 \pm 2.70$. In the second class, we have 1,087 individuals, comprising 547 males and 540 females. The sex ratio here is $101.30 \pm 4.07$. It is obvious that this difference must be regarded as accidental. When we restrict the "inbred" group to those individuals which were derived from the matings of full brothers and sisters, the sex ratio becomes somewhat larger, being $103.56 \pm 4.82$. But even here, the difference between this figure and that for the "outbred" group is much less than its probable error.

Various investigators have offered data which they believed to indicate an effect of close inbreeding upon the sex ratio. It is of interest (and quite characteristic of the literature of "sex determination" in general) that some writers have found a higher proportion of males in inbred strains, while others have found the females to be in excess.

Miss King (1918), from extended experiments on the white rat concludes that inbreeding *per se* does not affect the sex ratio. Her experiments yielded, however, one important positive result, namely that strains could be selected, giving preponderant numbers of male and female offspring respectively.

[1] It is necessary, with every brood, not only to look up the birth records but to trace out the pedigrees to some extent.

## Order of Birth.

Both the order of birth, and the age of the parents have been regarded by various writers as factors in determining sex. From our data it would be difficult to separate the influence of these two factors, had we reason to suspect that either of them had any effect upon the sex ratio. Since, however, our findings here are quite inconclusive, it is a matter of no importance that the possible influence of the two factors should be kept distinct.

In 196 cases, it happened that the same parents had two or more litters of young. The sex ratio for the 554 individuals of known sex comprised in the first broods[1] is found to be $91.70 \pm 5.26$. That for the 627 mice in the second broods is $104.91 \pm 5.65$. This would seem to be a considerable difference, but the numbers are small and the probable errors correspondingly high. We are not, therefore, warranted in attaching any significance to this difference. When we add the very limited number of third and fourth broods to the second ones and compute the sex ratio for the 738 mice of known sex belonging to these "later" broods, we have $103.31 \pm 5.48$. The probability of an actual difference in sex ratio between first and later broods is actually diminished by this procedure.

It is of some interest that the second broods averaged somewhat larger than the first, the mean figures being 3.39 and 3.05 respectively, based upon 196 broods of each class. This difference is nearly three times its probable error.

King and Stotsenburg report for the white rat a steadily decreasing sex ratio in passing from the first to the fourth litters borne by the same mothers, the first figure being 122.0, the last 74.5. The authors recognize, however, that the number of broods under consideration does not warrant any final conclusions on this point.

Punnett (1903) from an examination of Burke's "Peerage" concludes that the first born, in man, are predominantly males.

[1] By "first" broods we here mean the first which are known to have been borne by a given mother. Since, in more than a third of the cases, the mothers were wild mice, which had been trapped when nearly or quite mature, it is likely that many of these had already given birth to young. In such cases, the actual contrast is between *earlier* and *later* broods.

Newcomb (1904) likewise finds that "the first born child of any mother is more likely to be a male, in the proportion of about 8 to 7" (p. 28).

## Nutrition.

Our data on the much-discussed subject of the relation of nutrition to the sex ratio are extremely meager, though it seems worth while to include them in this report. Any such extreme effect as has been alleged by certain writers might have been expected to manifest itself, even in the limited numbers here considered.

For the purpose of testing the possible beneficial effect of including meat in the dietary of the stock, certain lots of mice, in three successive generations, were divided into two sections, one being given a rather liberal supply of chopped, boiled meat (commonly liver), in addition to the regular ration which was strictly vegetarian, the other (control) lot being restricted to the latter. The experiment was commenced at about the time when the first of the three generations referred to was mated. The meat diet was continued until the mice of the third generation were fully grown.

In all, there were 237 young of determined sex among the offspring of meat-fed parents (second and third generations). These gave a sex ratio of $104.31 \pm 9.13$. In the control lot were 228 young, giving a ratio of $96.56 \pm 8.61$. Here, again, it is obvious that the difference is not significant. The transfer of a very few individuals from the male to the female column (or vice versa) would be sufficient to reverse it.

Cuenot and Schultze (both cited by Morgan, 1907, pp. 385, 386) performed experiments upon rats and mice, respectively, with a view to testing the possible effects of feeding upon sex determination. In neither case were significant differences found between the sets of animals under comparison, but the numbers were very small and the results almost valueless statistically. More recently, Slonaker and Card (1918) have computed sex ratios for omnivorous and strictly vegetarian white rats, finding these to be 113.6 and 107.6 respectively. Since the number of individuals and other essential matters are not stated in the brief preliminary communication referred to, we must suspend judg-

ment on the significance of these figures. Most recent experiments with other animals have given negative results as regards the effect of diet upon the sex ratio.

## The Year.

We have already referred to the surprising fact that the sex ratios of mice born in different years may differ widely and significantly from one another. The following table gives the figures for all the years comprised in our records, with the exception of 1914, for which the data are very meager:

| | Males. | Females. | Ratio. |
|---|---|---|---|
| 1915 | 307 | 306 | 100.33 ± 5.44 |
| 1916 | 262 | 209 | 125.36 ± 7.82 |
| 1917 | 175 | 248 | 70.56 ± 4.70 |
| 1918 | 758 | 736 | 102.99 ± 3.67 |
| 1919 | 137 | 116 | 118.10 ± 10.03 |
| 1920 | 121 | 138 | 87.68 ± 7.37 |
| 1921 | 496 | 560 | 88.57 ± 3.68 |

As judged by the customary standard, some of these differences are highly significant. That, for example, between 1916 and 1917 is $54.80 \pm 9.1$, the difference being six times its probable error. The chances that this result was due to "accident" (*i.e.*, that no difference would have been found if our numbers had been indefinitely great) are only about 23 in a million. The difference between the ratio for 1916 and the ratio for the entire period ($97.37 \pm 1.93$) is 3.5 times its P. E., that between 1917 and the general ratio being 5.3 times its P. E. The difference between the largest figure (125.36) and the second smallest (87.68) is 3.5 times its P. E., that between the smallest figure (70.56) and the second largest figure (118.10) is 4.3 times its P. E. The difference between the second largest and the second smallest ratios is, however, only 2.5 times its P. E. Finally the difference between the ratios for the two years during which the greatest number of births occurred (1918 and 1921) is $14.42 \pm 5.2$, *i.e.*, it is 2.8 times its P. E.

The foregoing probabilities are, to be sure, not wholly cumulative, but the conclusion seems hardly avoidable that certain of them are real in the sense of not being due to errors or random

sampling. We must, of course, consider the possibility that these differences among the sex ratios of the various years are due to some of the other factors already considered. May they not, in part at least, be due to the unequal representation in these different years of broods born in months of high or of low sex ratio? Aside from the fact that these yearly differences are even more extreme than any of the seasonal ones, we have excluded this possibility by a procedure previously adopted in similar cases. The number born in each month of each year was multiplied by

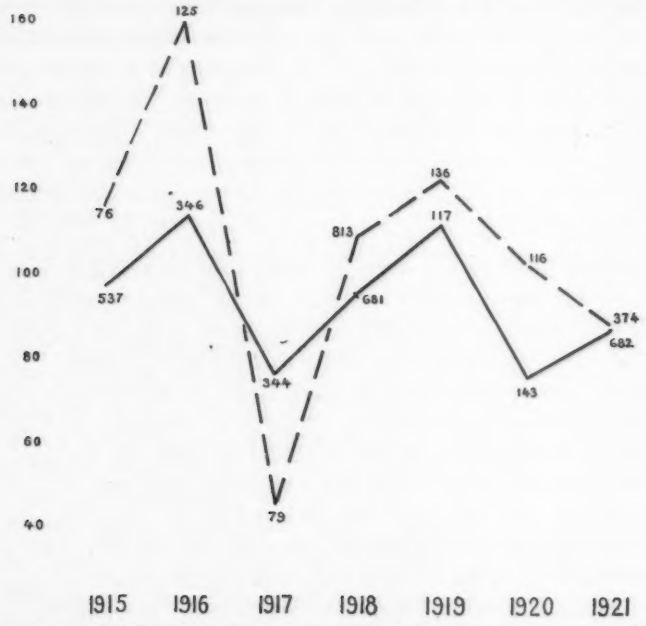


FIG. 7. Differences in the sex ratio during the different years comprised in our records. Continuous line = pure. Dotted line = hybrids.

the sex ratio for that month (based on the entire material). The weighted mean for the year in question was then computed. None of the yearly ratios thus obtained differed by more than 4.0 from the general ratio (97.37), showing that seasonal distribution cannot account for these yearly differences.

Nor are the latter to be accounted for by the possibly unequal distribution of pure and hybrid births. Figure 7 shows that the

same year-to-year fluctuations were undergone by both pure and hybrid stock, with the exception of the last (from 1920 to 1921).

When each of these years is divided into 3-month periods (February to April, etc.), we find that, on the whole, the years of low and high ratio show this tendency for each of the four "seasons." Graphs (not here reproduced) show that the "curve" for 1917, while undergoing closely similar fluctuations to that for the entire series, remains throughout far below the latter. Of the other two "low" years, 1921 remains throughout below the mean, while 1920 gives a lower ratio in three out of four of these periods. Of the two years which show ratios appreciably higher than the mean, each gives a "curve" which keeps far above the mean during three of the four seasons. The exceptions fall at different times in the two cases.

These considerations, while they add little to the evidence for the statistical reality of these yearly differences, show that the latter do not depend upon conditions which act primarily during any particular period of the year. To be sure, computations have been made which show that the departures of these yearly ratios from the mean condition occur preponderantly in the fall and winter months. This relation is probably due to chance, however, so that the figures need not be given here.

Similar differences have been recorded by a number of investigators between the sex ratios of different years for mankind, but so far as we know, these differences have not been discussed by them. They have been regarded as accidental, or at least as irrelevant and negligible. Thus in the study by the Pearls, already referred to, of the sex ratios of "pure" and "hybrid" races in the city of Buenos Aires, the authors confine their discussion to the apparently higher ratios which are found among offspring of mixed parentage. They quite overlook the fact, however, that the sex ratio of the entire population varies materially from one year to another, and that these yearly differences are in some cases both absolutely greater and statistically more certain than are those between the pure and hybrid elements of the population. Thus the ratio for the year 1900 is $97.0 \pm .90$, while that for 1904 is $105.4 \pm .95$. Each of these figures is based

upon over 20,000 births, and the difference is 6 times its probable error. Even greater and more "significant" differences are to be found in the yearly ratios of some of the separate population groups (in one case $8 \times$ P. E.), and it is of interest here to note that the years of high and low sex ratios do not tend to correspond in these various groups, but that they sometimes show exactly opposite conditions.

It is, of course, inconceivable that a difference of calendar year, as such, should have any more influence upon the sex ratios of mice or men than the phases of the moon or the conjunctions of the planets. It is likewise improbable that any mean difference in the weather from one year to the next, can be the responsible factor. For the meteorological differences between the most widely unlike years are small in comparison with the differences between the summer and winter seasons in any single year. But we have seen that these yearly variations of sex ratio may be even greater than the seasonal ones. Again, examination of the composition of the stock during these widely divergent years (say 1916 and 1917) gives no suggestion of a clue based upon considerations of this sort.

Huxley (1920) discusses a case in which a teleost fish produced, for nearly a year, three times as many females as males. Later, this ratio among the young produced changed to 2 females: 3 males for a few weeks, after which the numbers of the sexes became approximately equal and remained so for several years. Huxley believes it probable that in the first stage a certain proportion of the individuals having the zygotic formula XY became "somatic females" or "feminized males," and that such individuals produced X and Y eggs, which by fertilization gave rise in the next generation to an excess of males. A converse hypothesis might, of course, be invoked to explain the change in our stock, from a ratio of 125 in 1916 to one of 71 in 1917. But this is suggested merely as a remote possibility, justified only by the utter absence of any plausible scientific explanation to cover the case.

Finally, it should be urged that even highly improbable things sometimes happen, and that it is not impossible that our most

"significant" figures may result from chance. An illustration of this fact, drawn from the experience of the senior author, is worth recording here. Two series of mice had been subjected to a difference of treatment which even the most ardent believer in "sex determination" by physical agencies would not think of as being effective in this connection. The difference was that in one series the right sciatic nerve was cut, in the other the left. The first 9 broods of "right" parentage consisted of 14 females and no males. During the same period, 5 broods were born of "left" parentage, consisting of 10 males and 4 females. Had the attempt been made to influence sex by experimental procedure, the result would have seemed highly satisfactory. Thus, the chance of obtaining 14 individuals which were exclusively females is only one in 16,384, and the "significance" of the results is increased when we consider the high preponderance of males in the contrasted group. Experiences such as this lead one to demand higher statistical probabilities than are frequently accepted as convincing.

## Summary.

Data have been presented, based upon over 4,600 deer-mice of known sex, which were born and reared in captivity, under temperature conditions not far different from those existing in nature. The following results seem to be of most importance.

1. In size the broods ranged from 1 to 9, the mean of the 1,567 broods being 3.22.

2. The sex ratio for the entire lot was $97.37 \pm 1.93$. When we include only those broods in which no deaths are known to have occurred (nearly nine tenths of the whole) the figure becomes $98.01 \pm 2.07$. Broods known to have been incomplete give a ratio of $93.08 \pm 5.25$. In order to eliminate the effect of seasonal differences, the mean of the monthly means has been computed. This is 95.65. None of these figures can be regarded as differing significantly.

3. The probable errors employed throughout this paper are based upon a formula different from that which has been used by various previous writers. As a result, the errors here given are about twice as great as would formerly have been computed, and

the probabilities claimed by us are correspondingly lower. According to this safer criterion, some of the most interesting of our differences are not decisively "significant."

4. Considering our aggregate material, there seems to be a definite seasonal cycle in the proportion of males and females born. The sex ratio presents two annual maxima, in March–April and August–October, respectively; and two annual minima, in winter and summer, respectively. The lowest figure is 78.79 ($\pm$ 6.93), in November and the highest 113.04 ($\pm$ 8.04) in April. This difference is about 3¼ times its probable error, and would ordinarily be regarded as "significant." A well-marked biennial rhythm is shown by both pure and hybrid stock, taken separately, though the fall maximum occurs in different months in the two cases. On the other hand, when our material is subdivided in certain other ways, the results are in some cases highly contradictory. We cannot, therefore, regard the existence of a seasonal cycle in the sex ratio of *Peromyscus* as being proved conclusively by our data.

In any case, the position of these annual maxima and minima, as found by us, does not correspond with those which have been reported for the white rat or for man. Indeed, the conditions in *Peromyscus* are very nearly the reverse of those described by certain other authors.

5. Subspecific hybrids (1,722) give a mean ratio of 104.76 $\pm$ 3.41. Mice of "pure" race (2,930) give a mean ratio of 93.27 $\pm$ 2.32. Considering these figures alone, the difference is barely significant statistically, but its reality is borne out by various other considerations. Furthermore, such an effect of hybridization upon the sex ratio was to be expected in view of the findings of various other biologists, working upon widely different organisms. It is possible, also, that the several subspecies of *Peromyscus* differ *inter se* to some extent.

6. A positive correlation exists in our material between the size of the brood and the sex ratio. Broods containing one to three individuals give a mean sex ratio of 94.85 $\pm$ 2.94, ones containing four to nine individuals give a mean sex ratio of 102.42 $\pm$ 3.01. The magnitude of these probable errors raises the suspicion, however, that these differences are accidental, and

our doubts are further increased by the lack of a uniform gradation when the broods are grouped according to size.

7. When the number of each possible combination of males and females, in broods of each size, is compared with the number expected according to chance, the conformity is found to be, on the whole, very close. For example, the number of all-male and all-female broods (excluding broods of one) was 276, the "expected" number being 274. There is thus no preponderant tendency toward the production of homosexual litters, and thus no likelihood that polyembryony or true twinning is at all common in these animals.

8. In our material, the sex ratio is lower for the earlier broods (91.7) than for later broods of the same mother (103.3). The numbers are so small, however, that the difference is probably accidental.

9. Likewise, inbreeding and outbreeding seem to have had no influence upon the relative numbers of males and females, within the limited material available for this comparison.

10. Similarly negative results were obtained from a comparison of the offspring of meat-fed individuals with the offspring of those whose diet was strictly vegetarian. Here again, the numbers were too limited to permit us to regard this experiment as decisive.

11. The most significant result of all, statistically speaking, and one which is at present utterly inexplicable, is the fact that the sex ratios for the seven different years included in our records show a wide range of variation. The extreme figures are those for 1916 and 1917, the ratios being $125.36 \pm 7.82$ and $70.56 \pm 4.70$, respectively. These figures are based upon 471 and 423 individuals, respectively. The difference is $54.80 \pm 9.1$ (*i.e.*, difference $= 6 \times$ P. E.). The likelihood of obtaining such a result by "accident" is less than one in 40,000. We have furthermore determined that this difference is not due either to the seasonal distribution of births, to the preponderance of hybrid births in one year, as compared with another, or to the operation of any of the other factors previously considered.

Mention was likewise made of the occurrence of similar annual differences in the sex ratio of man as revealed by the tables of

Pearl and Pearl (1908). Some of these differences we have shown to be much more highly "significant"—according to accepted standards—than the differences between the pure and hybrid races with which these authors were concerned.

## Discussion.

In a field as well tilled as that of sex determination, it is both impracticable and undesirable for us to enter into any extensive review of the literature, and we offer no apology for the inadequate references to other workers which we have found it possible to include in the foregoing pages. Those who are unfamiliar with the literature of this field are referred to works by Geddes and Thomson (1914), Marshall (1910), Morgan (1913), and Doncaster (1914). The last two of these books are of particular value in this connection and supplement one another very nicely, since the viewpoint of the authors differs rather widely.

In recent years the view has been gaining ground that the sex of the individual is determined by the chromatin constitution of one or the other of the germ cells. In the majority of cases which have been investigated, it is believed that the differential factor is contained in the sperm cell, though there is evidence in a number of instances that the ovum is the gamete concerned. The cytological and genetic data upon which these conclusions are based are too well known to be discussed here. For many organisms the evidence is doubtless wholly convincing.

Many well-attested cases exist, however, in which the sex ratio is known to be correlated with certain external factors. Those who wish to universalize the chromosomal theory of sex determination, dispose of such cases by assuming the existence of either a differential mortality among the developing organisms, or a differential mortality among the sex-determining gametes, or some other factor favoring one class of gametes or zygotes, as compared with the other. It is assumed by such biologists that the sex of the organism is invariably predetermined in the nucleus of the fertilized egg, and is not subject to reversal by any influence that can be brought to bear subsequently.[1]

[1] Morgan (1913), for example, devotes a section of a chapter to "The Abandoned View that External Conditions Determine Sex," though there are reasons for believing that Professor Morgan does not take such an extreme position at the present time.

Other biologists, while granting this absolute predetermination of sex for some organisms, believe that in other cases the condition of the nucleus may be neutral in this regard, and that which of two potentialities shall finally prevail may be determined by chemical or physical influences acting upon the developing organism. There is, indeed, evidence which seems irreconcilable with the view that sex is always predetermined in the nucleus of the fertilized egg. We think, accordingly, that this second position is the safer one in the present state of our knowledge.[2]

As regards the data here reported for *Peromyscus,* we believe that (granting the reality of certain of the differences found) they lend themselves perhaps equally well to either of the above conflicting viewpoints. There may, for all we know, be seasonal differences in the relative production of male-producing and female-producing spermatozoa. Or, one kind may be more active at one time of the year than another. Or, the ova may so vary in their chemical composition that their attractive influence upon one or another type of spermatozoön varies with the season. Any or all of these things may be true, though there is not a scintilla of evidence that such is the case.

As regards the effect of crossing upon the sex ratio, this too, is reconcilable with either viewpoint. There might, in one way or another, be a preponderant selection of male-producing spermatozoa in hybrid fertilizations. On the other hand, the increased vigor which frequently accompanies hybridization might well influence the metabolism of the parents, and thus affect that of the developing fetuses.

When we come to those fluctuations in the sex ratio which are met with from one year to another, we must admit that their causes are utterly obscure. In the case of *Peromyscus,* it is difficult to conceive of any scientific explanation whatever for the fluctuations found. Even when we admit the likelihood that environmental conditions may play a direct or indirect part in sex determination, it is hard to see how the relatively slight differences which distinguish one year from another could bring about a greater effect than the vastly larger differences which distinguish the seasons of a single year. The calendar year, as such, would

[2] This is the viewpoint adopted by Doncaster (1914, Chapter X.).

seem to be as devoid of causal efficacy in this matter as are the planetary influences which are invoked by the astrologists.

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